1996

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OSTEOPONTIN (OPN): STRUCTURAL ANALYSIS OF THE MOUSE *OPN* GENE AND FUNCTIONAL ANALYSIS OF THE OPN PROTEIN IN MALIGNANCY

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
December 1995

ISBN 0-612-09852-4

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ABSTRACT

Osteopontin (OPN) is an integrin-binding cell adhesion protein, that is expressed in many normal cells and tissues, and that is associated with a number of pathological conditions, including cancer. The work presented in this thesis addresses both structural studies of the *opn* gene, and functional studies of the OPN protein, in an attempt to improve our understanding of OPN.

Two conflicting gene structures of the mouse *opn* gene had been reported that put into question the location of the promoter, the transcriptional start site, and exon 1 of the mouse *opn* gene. Analysis of the expressed messages predicted by the two reported gene structures has established the correct gene structure of mouse *opn*. Furthermore, this analysis has established that the *opn* transcript (mRNA) is similar in mouse fibroblasts, mouse epithelial cells, and mouse macrophages, an important observation since the conflicting *opn* gene structures were derived from epithelial and macrophage cells.

The functional role of OPN in any of the physiological or pathological contexts in which OPN is found, is still unknown. Frequently, OPN has been associated with conditions of transformation, including being over-produced by *ras*-transformed NIH 3T3 cells. Analysis of the levels of *opn* mRNA and OPN protein produced in a series of *ras*-transformed NIH 3T3 cells has demonstrated that *opn* mRNA and OPN protein levels correlate with the levels of *ras* expression and with the metastatic potential of the cells. This finding raised the possibility of an active role for OPN in the development of a malignancy.

To determine whether OPN contributes functionally to the malignant properties of tumor cells, antisense *opn* RNA was expressed in metastatic, *ras*-transformed NIH 3T3 cells. Two independent clones that expressed antisense *opn* RNA *in vitro* were significantly reduced in their ability to form primary tumors and metastases *in vivo*. Primary tumors did arise only after expression of antisense *opn* RNA was lost from the tumor cells, independently in five individual mice. These results are consistent with a functional role for OPN in malignancy, and are the first to show expression of antisense *opn* RNA associated with decreased malignancy.

To my family

ACKNOWLEDGEMENTS

I would like to acknowledge the people who, each in their own way, have contributed to making this thesis a reality.

Most of my thanks and deepest gratitude go out to my supervisor Dr. Ann Chambers. By taking me on as a graduate student, Dr. Chambers has provided me with an opportunity few places of work provide, the opportunity to grow as a person, intellectually, emotionally, mentally, and socially. In the true sense of the word, Dr. Chambers has been a mentor to me, teaching me the skills of scientific thinking and scientific conduct. Dr. Chambers has taught me valuable technical skills such as writing effective papers, looking for and finding sources of funding, and giving clear oral presentations. However, she has also taught me to think critically, to believe in myself, and most importantly, to never give up. Thanks to Dr. Chambers I have replaced the words "I can't" with "somehow I will". Dr. Chambers has been very supportive in every aspect of my post-graduate training and has allowed me to learn the lessons of graduate school at my own pace. I must admit that nothing in my training as a graduate student came easy, but I have never valued a learning experience as deeply as this one. I could not have wished for a better mentor than Dr. Chambers, and I don't.

I would also like to acknowledge the greatly appreciated contributions of my graduate advisors, Dr. Michael Clarke and Dr. James Koropatnick to my thesis project. Their encouragement, advice and resourcefulness for new ideas has been invaluable to this thesis. I would like to thank Dr. Clarke for performing the Gen Pep database search that was required as part of this work. Special thanks go out to Dr. Koropatnick for reading this thesis, and for his never-ending enthusiasm to write letters of recommendation for me.

My thanks also go out to Dr. David Denhardt for providing some of the materials used in this study, for reading and providing helpful suggestions on manuscripts and parts of this thesis, for writing letters of recommendation, and for giving advice and encouragement.

I would also like to express my thanks to members of the Chambers lab, Mrs. Sylvia Hill, Mrs. Charulata Hota, Dr. Alan Tuck, and Dr. Lynn Weir, as well as other members of the Cancer Research Laboratories, Debbie Aristone, Don Van Meyel, and

Cathy Nutt for their friendship and their practical jokes, which have made my years as a graduate student sometimes unpredictable, but enjoyable. Special thanks go out to Mrs. Sylvia Hill for her technical advice, for teaching me all necessary lab skills, and for her assistance in the chick embryo assay. Thank you to Dr. Alan Tuck for teaching me how to run RNA gels and perform Northern blot analysis, and thank you to Dr. Lynn Weir for reading this thesis and giving helpful suggestions.

Finally, I would like to thank my family, who have taught me the values of hard work and honesty, to give a job the best I can, and most of all to never quit and to finish a job that I have started. My family has always stood by me, believed in me, and given me support and encouragement in everything I do.

Thank you to all of you.

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LIST OF ABBREVIATIONS

A	adenine
AP-1	activator protein 1
apc	(familial) adenomatous polyposis coli gene
Arg	arginine
asOPN	antisense OPN
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
α	alpha
BAG-75	bone acidic glycoprotein
BALB/c	an inbred mouse strain
B.C.	before Christ
β	beta
bis	methylene-bis-acrylamide
bp	base pair(s)
brca1	breast cancer 1 gene
BSP	bone sialoprotein
C	cytosine
Ca^{2+}	calcium
$CdCl_2$	cadmium chloride
CDK	cyclin-dependent kinase
cDNA	complementary DNA
cf	compare with figure
cm^2	centimeter squared
cNOS	constitutive nitric oxide synthase
CO_2	carbon dioxide
cpm	counts per minute
CS	calf serum
C3H/RV	an inbred mouse strain
C3H/He	an inbred mouse strain

°C	degrees centigrade
dcc	deleted in colon carcinoma gene
dCTP	deoxycytidine 5′-triphosphate
DSP	dentin sialoprotein
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ERK2	extracellular-signal regulated kinase 2
Eta-1	early T-lymphocyte activation 1
EtOH	ethanol
FBS	fetal bovine serum
γ	gamma
g	gravity
G	guanine
g/L	grams per liter
GAP	GTPase-activating protein
GDP	guanosine 5′-diphosphate
GDS	guanine nucleotide dissociation stimulator
Glu	glutamic acid
Gly	glycine
GRGDS	glycine-arginine-glycine-aspartic acid-serine
GTP	guanosine 5′-triphosphate
h	hour
HA	hydroxyapatite
HBSS	Hanks' balanced salt solution
HCl	hydrochloride
hnRNA	heteronuclear RNA

ICR	an inbred mouse strain
IgG	immunoglobulin G
IgM	immunoglobulin M
iNOS	inducible nitric oxide synthase
i.v.	intravenous
IVVM	intravital videomicroscopy
kb	kilo base(s)
kbp	kilo base pair(s)
kD	kilo dalton(s)
KNRK	Kirsten sarcoma virus-transformed NRK cells
λ	lambda
LB broth	Luria-Bertani broth
LPS	lipopolysaccharide
LTR	long terminal repeat
M	molar
μCi	microcurie
μg	microgram(s)
μm	micrometer(s)
μM	micromolar
ml	milliliter
mM	millimolar
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
min	minute(s)
MMTV	mouse mammary tumor virus
MOPS	3-(N-Morpholino)propanesulphonic acid
M_r	relative molecular weight
MRE	metal response element
MRL/lpr	an inbred mouse strain
mRNA	messenger ribonucleic acid

MT	metallothionein
MT-I	metallothionein-one
MT-IIA	metallothionein-two A
NaCl	sodium chloride
NaOH	sodium hydroxide
NB1 (nb1)	neuroblastoma 1
NF1 (nf1)	neurofibromatosis 1
NF2 (nf2)	neurofibromatosis 2
NK	natural killer cells
NO	nitric oxide
NP-40	Nonidet P40
NRK	normal rat kidney cells
nt	nucleotide
OPN	osteopontin protein
opn	osteopontin gene or mRNA
ORF	open reading frame
P	phosphate
PBS	phosphate buffered saline
PDA	piperazine di-acrylamide
PDGF	platelet-derived growth factor
PEA-3	polyoma enhancer activator 3
PKC	protein kinase C
PTE	proximal tubule epitheliai cells
RB (rb)	retinoblastoma
RGD	arginine-glycine-aspartic acid
Ric	*Rickettsia tsutsugamushi (RT)*
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase H	ribonuclease H
RNase III	ribonuclease III
RNase P	ribonuclease P

RNase A	ribonuclease A
ROS	rat osteosarcoma cells
S	sulphur
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SLE	systemic lupus erythematosus
SMC	smooth muscle cells
SPP-1	secreted phosphoprotein 1
SSPE	sodium sodium phosphate ethylenediaminetetraacetic acid
SSC	sodium sodium citrate
T	thymine
T cells	T lymphocytes
TE	Tris-EDTA buffer
TG	transglutaminase
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAP	tartrate-resistant acid phosphatase
Tris	tris(hydroxymethyl)-aminomethane
TSM	Tris sodium chloride magnesium chloride
U	uracil
v/v	volume/volume
WT1 (wt1)	Wilms' tumor
X	any amino acid

CHAPTER 1
INTRODUCTION

1.1 OVERVIEW

Cancer is an old disease. It was described as early as 1770 B.C. (Mainwaring, 1986). Only recently has our understanding of this disease improved significantly, mainly due to advances in molecular biological techniques. It is widely accepted now that cancer is a genetic disease which progresses by sequential alteration of genes in individual tumor cells. The introduction of this thesis will therefore focus on: (1) discoveries and hypotheses that have shaped the current view of cancer development and progression, (2) a review of cellular and molecular steps that are involved in the generation of a malignancy, (3) a discussion of genes that are involved in tumor progression, and (4) a review of one molecular biological technique, antisense technology, that was employed in this study.

1.2 CANCER DEVELOPMENT AND PROGRESSION

"Tumor initiation occurs by an alteration in a single, previously normal cell, that provides the cell with a growth advantage over adjacent normal cells. As tumors progress they become more aggressive in their behavior and more malignant in their characteristics" (Nowell, 1976).

The clonal origin of most neoplasms is a concept that is supported by a substantial collection of experimental data and that has gained wide acceptance and recognition (reviewed by Nowell, 1976; 1983; 1986; 1990; Fialkow, 1979; Cairns, 1975; Arnold *et al.*, 1983). Tumor cells typically exhibit an increased growth rate compared to normal cells, and undergo morphological changes that are indicative of loss of differentiation. Their loss of dependence on anchorage and serum, lack of responsiveness to hormones, decreased antigenicity, and acquisition of invasiveness, metastatic ability, and drug-resistance mark the progressive escape of these cells from growth control mechanisms that regulate normal cells. Tumors and tumor cell populations are also marked by their inherent genetic instability and cellular heterogeneity, which can contribute to tumor progression and to the development of drug-resistant and metastatic cells (reviewed by Nowell, 1983; Nicolson, 1987; Volpe, 1988). Nowell's (1976) model of "the clonal

evolution of tumor cell populations" represents a significant milestone in our understanding of tumor development and progression, since it translated the accumulated scientific and clinical data into a testable working hypothesis. This theory explains tumor development and progression, from a single altered cell (clone), in terms of the sequential selection of variant subpopulations from this original clone. The existence of these distinct subpopulations of tumor cells demonstrates the striking heterogeneity found in many tumors. The well-documented genetic instability of tumor cells increases the probability of accumulating additional genetic lesions with time, and provides a mechanism to generate cellular diversity within tumors. A particular subpopulation will dominate the tumor mass until a more favorable clone with decreased growth restraint emerges. This process continues to produce variants that are invasive, metastatic, and/or drug-resistant, and ends when either cancer therapy is successful at eliminating all cancer cells or when the patient's body can no longer sustain itself.

Another significant advancement in the study of cancer was the discovery of the involvement of oncogenes and tumor suppressor genes in the development of cancer, which demonstrated clearly that cancer was a genetic disease. Cancer had long been suspected to have a genetic basis, a belief that was founded on: (1) the hereditary predispositions to some cancers, (2) the detection of damaged chromosomes in cancer cells, (3) the susceptibility to cancer development of cells impaired in their ability to repair damaged DNA, and (4) the ability of mutagens to function as carcinogens (reviewed by Bishop, 1987; 1989; 1991). The first evidence for the involvement of genes in the development of cancer, in fact, came from the identification of transforming genes (oncogenes) in acutely-transforming retroviruses (reviewed by Varmus, 1989; Bishop, 1987; 1989).

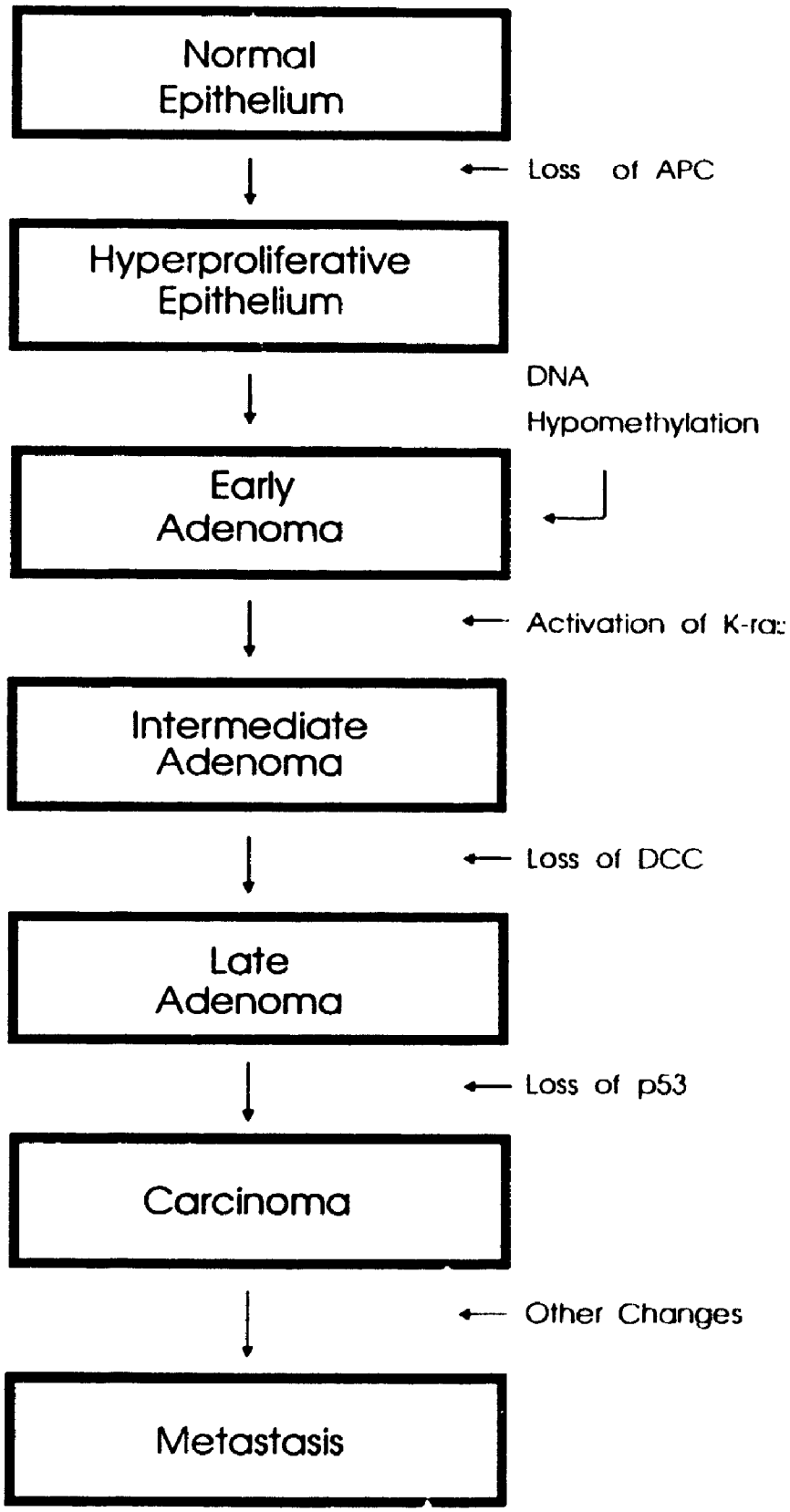
Oncogenes are mutated cellular genes that act in a dominant manner to transform cells and produce tumors in animals and humans (reviewed by Spandidos and Anderson, 1989; Bishop, 1989; Brison, 1993). Their protein products function as positive regulators of cell growth and proliferation to promote the malignant properties of transformed cells. The cellular genes from which oncogenes are derived, the proto-oncogenes, are important components of cellular pathways that regulate cell communi-

cation, cell growth, division, and differentiation.

Tumor suppressor genes are cellular genes whose protein products are also essential components of cellular signal transduction pathways, but they function as negative regulators of cell growth to control cell proliferation. Tumor suppressor genes and their involvement in cancer were identified through: (1) somatic cell hybridization experiments between normal and transformed cells, (2) the study of familial cancers, and (3) loss of heterozygosity studies (Levine and Momand, 1990; Marshall, 1991; Weinberg, 1991). In cancer, these genes are frequently inactivated, either by deletion or mutation (reviewed by Marshall, 1991; Weinberg, 1991; Levine, 1993; Knudson, 1993).

Cancer has long been thought to be a multistep process that progresses with time and moves through qualitatively different stages (Foulds, 1957; Peto *et al.*, 1975; Cairns, 1975). The realization that mutated proto-oncogenes and functionally-deleted tumor suppressor genes are involved in the generation of a malignancy has permitted scientists to shed some light on the molecular genetic mechanisms that underlie the processes of tumor development and progression. The malignancy that is best characterized at the genetic level is that of colorectal carcinoma. Colorectal tumors progress through easily recognizable clinical stages for which the nature of some mutations has been identified (Fearon and Vogelstein, 1990) (see Figure 1.2). The genetic model of colorectal tumorigenesis proposed by Fearon and Vogelstein (1990) is consistent with the hypothesis that colon cancer does not arise from a single mutation, but rather is the result of multiple, successive genetic alterations which include both mutations in oncogenes (*ras*, *myc*, *neu*, *myb*) (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988; D'Emilia *et al.*, 1989; Alitalo *et al.*, 1983; 1984; Finley *et al.*, 1989) and mutations in or loss of tumor suppressor genes (*apc*, *dcc*, *p53*) (Vogelstein *et al.*, 1988; Baker *et al.*, 1989; Nigro *et al.*, 1989; Fearon *et al.*, 1990; Fearon and Vogelstein, 1990; Kinzler *et al.*, 1991; Joslyn *et al.*, 1991; Vogelstein and Kinzler, 1993). Alterations that are responsible for progression of a colon carcinoma to an invasive and metastatic phenotype have yet to be identified, but research in this area is expanding at an increasing rate.

Figure 1.2. A genetic model for colon tumorigenesis. Colorectal tumors progress through easily recognizable clinical stages, which are shown in boxes. A series of genetic alterations involving oncogenes and tumor suppressor genes accompany the clinical stages as colorectal tumors progress from early to late stages of tumorigenesis. The order of the genetic changes is not invariant, and accumulation of these changes, rather than their order with respect to one another, seems most important. (Figure modified from Fearon and Vogelstein, 1990).


Normal Epithelium
Loss of APC
Hyperproliferative Epithelium
DNA Hypomethylation
Early Adenoma
Activation of K-ra
Intermediate Adenoma
Loss of DCC
Late Adenoma
Loss of p53
Carcinoma
Other Changes
Metastasis

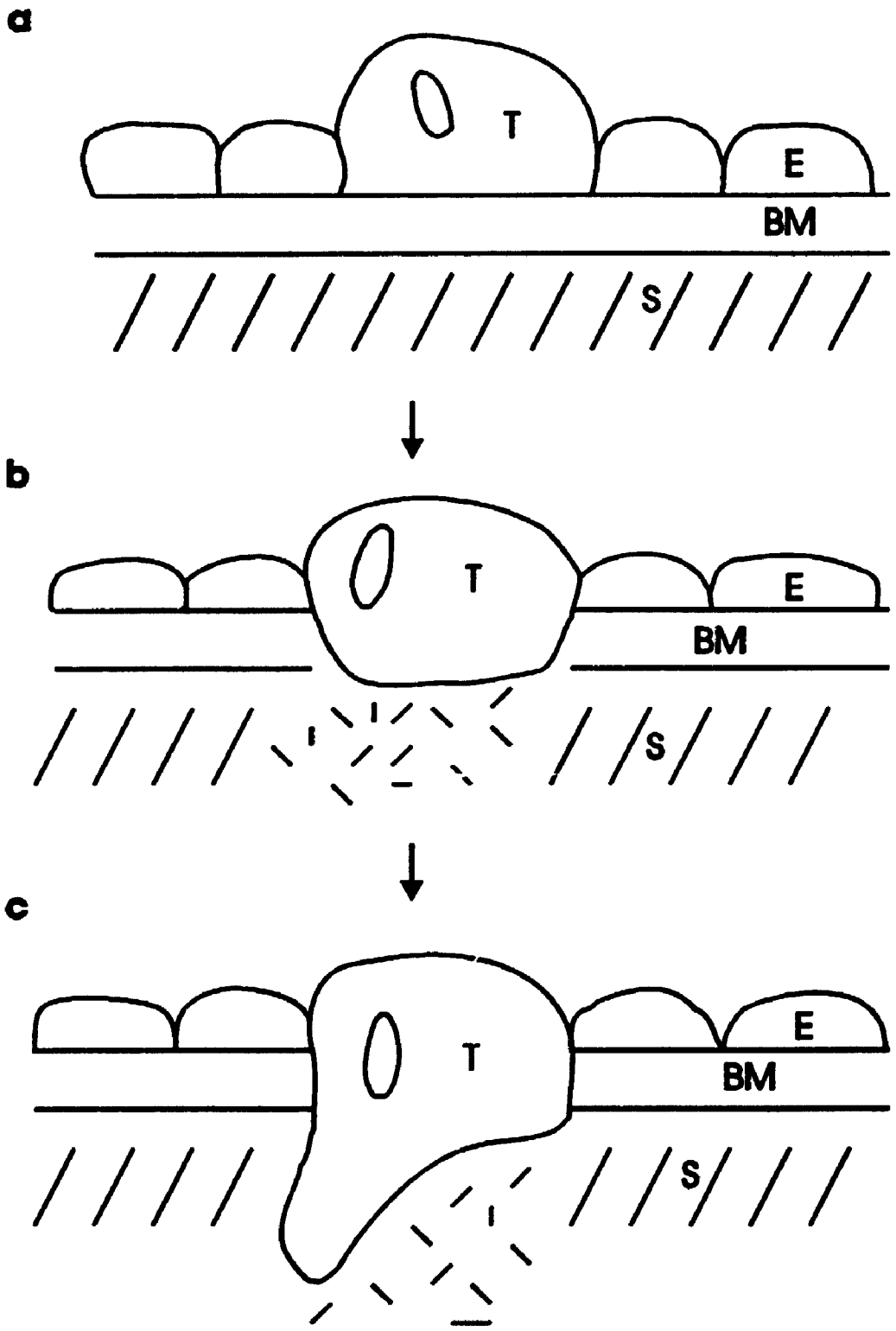
1.3 CELLULAR AND MOLECULAR STEPS IN METASTASIS

During the course of tumor progression some tumor cells acquire the ability to invade surrounding tissue, enter the circulation, and form secondary tumors at distant sites. These abilities to invade and metastasize define malignancy and represent the aspect of tumor progression that is of greatest clinical significance. Most cancer patients die from metastatic disease rather than the primary tumor. If cancer cells did not metastasize, removal of the primary tumor would result in a cure for the majority of patients. Therefore, to control or prevent metastasis is an important challenge in cancer treatment.

The first step taken by a tumor cell in the process of metastasis is to invade the surrounding tissue to reach a blood vessel. A model depicting extracellular matrix invasion as an organized sequence of three successive steps was devised by Liotta *et al.* (1991) (see Figure 1.3). According to this model invasion requires: (1) the attachment of the tumor cell to components of the extracellular matrix, (2) local proteolysis of the extracellular matrix, and (3) tumor cell locomotion. Individual components of this very complex process are under intense scientific investigation, and involve cell-cell and cell-matrix adhesive interactions, the local release of degradative enzymes and their inhibitors, as well as changes in the tumor cell cytoskeleton to achieve migration of the tumor cell towards a capillary (reviewed by Stracke and Liotta, 1992; Stetler-Stevenson *et al.*, 1993; Zetter, 1993; Lester and McCarthy, 1992; MacDonald and Steeg, 1993; Aznavoorian *et al.*, 1993). At each stage of the metastatic process, tumor cells are subject to attack by the host's immune system (Miller, 1993).

The recently developed technology of intravital videomicroscopy (IVVM) has allowed scientists to observe directly the fate of injected tumor cells, and has put into serious question previous theories regarding the fate of tumor cells (Chambers *et al.*, 1992a; MacDonald *et al.*, 1992; Morris *et al.*, 1993; Chambers *et al.*, in press). For example, biomechanical destruction of tumor cells in the circulation has been reported to account, in part, for the metastatic inefficiency of cancer cells (Weiss, 1987; 1991; Weiss and Schmid-Schönbein, 1989; Weiss *et al.*, 1992). However, IVVM has revealed that most cancer cells stay intact in the circulation, and once arrested in a blood vessel

Figure 1.3. The three-step model of extracellular matrix invasion by a tumor cell. (A) Step 1: Attachment. The tumor cell attaches to components of the extracellular matrix. (B) Step 2: Local Proteolysis. Tumor cell-associated proteases degrade the extracellular matrix near the site of tumor cell attachment. (C) Step 3: Locomotion. The tumor cell migrates into the region of the matrix that has been modified by proteolysis. Invasion of the extracellular matrix may continue by cyclic repetition of these three steps. *T*, tumor cell; *E*, endothelial cell; *BM*, basement membrane; *S*, stroma. (Figure modified from Liotta *et al.*, 1991).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due to size restriction, extravasate readily (Morris *et al.*, 1994; Koop *et al.*, 1994). It appears that it is the steps following extravasation of the cancer cells that are critical in determining whether a secondary tumor will grow. Koop *et al.* (1994) and Morris *et al.* (1994) have shown that poorly metastatic cells were compromised in their ability to grow post-extravasation when compared to cells of high metastatic potential. These observations suggest that growth after extravasation may be the rate-limiting step responsible for metastatic inefficiency, and offer a new target that can be explored for anti-cancer therapy.

The fact that specific tumor cells preferentially metastasize to specific organs suggests that local growth factors and their inhibitors as well as interactions with cells and extracellular matrix components significantly influence the ability of a tumor cell to establish metastatic tumors at the new site (Nicolson, 1988; 1991; 1993). Early in the process of tumor metastasis, tumor cells are believed to be responsive to paracrine and autocrine growth factors, as well as to growth inhibitors (Nicolson, 1993). The presence or absence of such factors may affect the rate of tumor growth, and could in part account for the observed dormancy of some tumors (Alexander, 1983; Schirrmacher, 1985; Demicheli *et al.*, 1994; Michelson and Leith, 1994). During later stages of tumor spread, as cells have acquired more growth autonomy as a result of additional genetic or biochemical changes, metastatic growth resumes and widespread metastases occur as tumor cells may be less responsive to paracrine signals.

Angiogenesis, the generation of new blood vessels from existing ones, also influences the ability of a metastasized cell to form a secondary tumor at the new location. Indeed, angiogenesis is required for both the growth and survival of the primary tumor and growth of the metastatic colonies (Folkman, 1992). The stimulus to undergo angiogenesis is likely to be tumor cell-derived or tumor cell-induced and acts either directly on endothelial cells or activates local inflammatory cells to induce angiogenesis (Weinstat-Saslow and Steeg, 1994). The dependence of tumor growth on neovascularization makes it an attractive target for anti-cancer therapy (Weinstat-Saalow and Steeg, 1994; Fidler and Ellis, 1994).

Research on tumor development, progression and metastasis has contributed to

our improved understanding of the cellular and molecular genetic events that occur during the development of a malignancy. At the same time, this research has shed light on many regulatory mechanisms and cellular pathways that govern normal cell behavior.

1.4 GENES INVOLVED IN TUMOR PROGRESSION

1.4.1 Oncogenes and Tumor Suppressor Genes

The coordinated growth of cells in a multicellular organism is mediated through complex pathways in which signals are propagated from the outside of the cell to specific targets within the cell, such as the cytoskeleton or the nucleus. These pathways are tightly regulated to maintain the integrity of the organism. Cancer is a genetic disease in which successive genetic alterations in key regulatory genes, proto-oncogenes and tumor suppressor genes, lead to a breakdown of the cellular growth control mechanisms and result in uncontrolled cell division and growth.

Proto-oncogenes comprise a class of genes that act in a dominant fashion to promote cell growth in response to an appropriate external signal. The protein products encoded by proto-oncogenes are localized at various points throughout cellular signal transduction pathways. Their localization provides information about their role. Some function as extracellular growth factors (SIS, INT-2, HST) and initiate the signal transduction cascade by binding to cell surface receptors, some of which are also proto-oncogenes (ERBB, FMS, MET, NEU, KIT, TRK) (Hunter, 1991; Bos, 1992; Perkins and Vande Woude, 1993; Schmandt and Mills, 1993). Other proto-oncogene products function downstream from the growth factor-growth factor receptor complex to relay the signal to: (1) the cytoskeleton which affects cell shape and motility, (2) the nucleus where regulation of the cell cycle and transcription are affected, or (3) other points such as the plasma membrane where ion channels or cell interactions may be altered. Some of these second messengers function as membrane-associated non-receptor tyrosine kinases (SRC, ABL, YES, LCK, FGR, FES/FPS, FYN, HCK), GTP-binding proteins (RAS, GSP, GIP2), or cytoplasmic serine/threonine kinases (RAF, MOS, BCR, PIM1) (Hunter, 1991; Bos, 1992; Perkins and Vande Woude, 1993; Schmandt and Mills, 1993). The exact sequences and events that constitute many of these signaling pathways are not known and

are only beginning to be unraveled. However, it is known that a number of proto-oncoproteins function in the nucleus as transcription factors (FOS, JUN, MYC, MYB, REL, ETS, SKI, ERBA) (Hunter, 1991; Bos, 1992; Perkins and Vande Woude, 1993; Schmandt and Mills, 1993). It is becoming increasingly clear that components of the cell cycle machinery may be directly involved in the generation of cancers. Mutations in or over-expression of some of these cell cycle components, such as cyclins D1, D2, D3, cyclin E, cyclin A, and cyclin-dependent kinase 4 (CDK4) have been reported in some cancers or have been associated with some aspect of the transformed phenotype, making these gene products likely proto-oncogene candidates (Hunter, 1993; Sherr, 1993; Hinds, 1994; Keyomarsi *et al.*, 1994).

For most human cancers, the majority of mutated genes are tumor suppressor genes (Knudson, 1993). Tumor suppressor genes, in contrast to proto-oncogenes, function to suppress cellular proliferation. Two independent mutational events (the "two-hit" hypothesis) are required for a tumor suppressor gene to be functionally deleted (Knudson, 1971). This phenomenon arises most commonly by a mutation in one allele followed by a loss of heterozygosity (chromosome loss or deletion) leaving only the mutated allele (Levine, 1993). A germline mutation in one allele of a tumor suppressor gene results in all somatic cells carrying this defect, and predisposes the individual to tumor development (Knudson, 1971). Fewer tumor suppressor genes than proto-oncogenes have been identified, and less is known about their function. Among some of those identified (i.e. *p53*, *rb*, *wt1*, *nf1*, *nf2*, *apc*, *nb1*, *brca1*), *p53* is by far the most studied tumor suppressor gene (Tominaga *et al.*, 1992; Kastan *et al.*, 1995).

p53 was initially identified as an oncogene based on transfection of mutated (assumed normal) *p53* into cells causing transformation (Lane and Crawford, 1979; Linzer and Levine, 1979; Deleo *et al.*, 1979). However, the wildtype P53 protein was soon identified and shown to suppress the tumorigenic phenotype by functioning as a cell cycle control protein (Hinds *et al.*, 1989; Diller *et al.*, 1990; Baker *et al.*, 1990; Martinez *et al.*, 1991). It has now been shown that wildtype P53 functions to inhibit cell cycle progression by arresting the cells in the G1 phase of the cell cycle in response to DNA damage or by inducing apoptosis (Kastan *et al.*, 1991; Kastan *et al.*, 1992; Kuerbitz

et al., 1992; Lowe *et al.*, 1993; Clarke *et al.*, 1993; Lotem and Sachs, 1993). Mutation of the P53 protein is a very frequent alteration in many human cancers (Hollstein *et al.*, 1991; Nigro *et al.*, 1989; Takahashi *et al.*, 1989). Its role in cell cycle control following DNA damage and its ability to induce apoptosis could account for the frequent functional loss of P53 in many human cancers.

1.4.2 The *ras* Oncogene

Mammalian *ras* genes are members of the superfamily of GTP-binding proteins, which function as molecular switches in many cellular signal transduction pathways (reviewed by Cox and Der, 1992; Lowy and Willumsen, 1993). Three functional human *ras* genes (H-*ras*, K-*ras*, and N-*ras*) encode four structurally related proteins (H-RAS, K4A-RAS, K4B-RAS, N-RAS) (Barbacid, 1987). The genes encode proteins of low molecular weight, 21 kD, with K-*ras* expressing two proteins as a result of alternative splicing of exon 4 (McGrath *et al.*, 1983).

RAS proteins are localized to the inner face of the plasma membrane, bind GTP and GDP, and possess intrinsic GTPase activity (Cox and Der, 1992). Under normal cellular conditions, RAS is present in its inactive, GDP-bound conformation. Upon stimulation with an appropriate signal, guanine nucleotide exchange (GDS) proteins promote replacement of GDP with GTP to switch RAS to its active, GTP-bound conformation (Khosravi-Far and Der, 1994). GTPase-activating proteins (GAPs) stimulate hydrolysis of GTP to GDP to return RAS to its inactive state (Khosravi-Far and Der, 1994).

RAS undergoes a series of post-translational modifications at the C-terminus prior to localization at the plasma membrane, which include: farnesylation, proteolysis, methylation, and palmitoylation (reviewed by Cox and Der, 1992; Lowy and Willumsen, 1993). These modifications are critical to the biological activity of RAS, since they enable the protein to localize to the cytoplasmic side of the plasma membrane, where the protein functions.

Ras genes were first identified as the transforming genes (v-*ras*) of acute transforming retroviruses (Harvey, 1964; Ellis *et al.*, 1980). Later, it was recognized

that normal cells contained genes (H-*ras*, K-*ras*, N-*ras*) that were homologous to v-*ras* (Der *et al.*, 1982; Parada *et al.*, 1982; Goldfarb *et al.*, 1982; Shimizu *et al.*, 1983). Activation of these cellular *ras* genes by a single point mutation resulted in oncogenic transformation of many cells (Barbacid, 1987). Furthermore, mutated proto-oncogenes were identified in many human and animal tumors (Bos *et al.*, 1987; Bos, 1989; Adams and Cory, 1991; Mangues and Pellicer, 1992). The most common activating point mutations in *ras* genes isolated from tumors occur in codons 12, 13, or 61 (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Yuasa *et al.*, 1983; Taparowsky *et al.*, 1983), although others have been reported. Activating mutations inhibit the protein's intrinsic GTPase activity, locking it in the activated, GTP-bound conformation. The activated conformation is a signal for the cell to continue proliferating, even in the absence of an external stimulus. Constitutive activation of the signaling pathways regulated by RAS results in increased cell proliferation and transformation of the cell (McCormick, 1989; Hall, 1990).

Recently, a biochemical function for RAS was reported (Hall, 1994). GTP-bound, activated RAS functions as a membrane-targeting signal for the mitogen-activated protein kinase kinase kinase (MAPKKK), RAF. Once localized to the membrane, RAF becomes activated by autophosphorylation or possibly protein kinase C (PKC) and initiates activation of the RAF-ERK2 (extracellular-signal regulated kinase 2) kinase cascade (Burgering and Bos, 1995). Mutated RAS activates this pathway and likely other growth promoting pathways constitutively, even in the absence of an external stimulus, resulting in uncontrolled cell proliferation.

1.4.3 Downstream Effector Genes

Both proto-oncogenes and tumor suppressor genes exert their function by affecting other cellular genes involved in signal transduction, transcriptional activation, proliferation, differentiation, adhesion, invasion, and other cellular processes (Aoyama and Klemenz, 1993; Bortner *et al.*, 1993; Chambers and Tuck, 1993). The most intensely studied oncogene is *ras*, and signaling events downstream of RAS are beginning to be elucidated (Khosravi-Far and Der, 1994). Transformation of cells with a *ras* oncogene results in the activation of many kinases, including RAF, MAP kinases, and

protein kinase C (PKC) (Roberts, 1992; Leevers and Marshall, 1992; Morrison *et al.*, 1988; Morris *et al.*, 1989). Activation of the RAF-ERK2 kinase signaling pathway mediates transduction of the signal to the nucleus, where it may lead to induction of genes such as *myc*, or *fos* and *jun*, whose protein products constitute transcription factors that can, in turn, activate the expression of other genes (Kyriakis *et al.*, 1992; Liaw *et al.*, 1993; Pulverer *et al.*, 1991; Seth *et al.*, 1992; Baker *et al.*, 1992; Stacey *et al.*, 1987a, 1987b). In NIH 3T3 cells, transformation with a *ras* oncogene results in proliferation of the cells, while in PC12 pheochromocytoma cells and F9 embryonal carcinoma cells, activated RAS triggers cell differentiation (Feramisco *et al.*, 1984; Stacey and Kung, 1984; Bar-Sagi and Feramisco, 1985; Sassone-Corsi *et al.*, 1989; Yamaguchi-Iwai *et al.*, 1990). RAS has also been proposed to regulate the function of RAC and RHO, members of the RAS-related RHO family of proteins, which have been implicated as regulators of actin cytoskeletal organization (Hall, 1992; Downward, 1992). As such, RAS and RHO proteins may be important in the regulation of cell shape, motility, and cell-cell interactions and influence cell adhesion and invasiveness of tumor cells (Khosravi-Far and Der, 1994). Chambers and Tuck (1993) have shown that expression of genes such as cathepsin L, cathepsin B, type IV collagenase, osteopontin, and calcyclin is up-regulated in *ras*-transformed cells, while expression of cystatin, *timp-1*, *timp-2*, *jun*, *fos*, and *Rb* (retinoblastoma) is decreased, consistent with a role for RAS in the regulation of these genes. Induction or suppression of *jun* and *fos* gene expression in response to *ras* appears to differ with the amount of *ras* expression and the cell type (Chambers and Tuck, 1993). Constitutive expression of *ras* in NIH 3T3 cells led to decreased expression of *jun* and *fos* (Tuck *et al.*, 1991), while transient expression of *ras* resulted in increased expression of *jun* and *fos* (Stacey *et al.*, 1987b; Sistonen *et al.*, 1989).

Even less is known about the downstream effector genes of tumor suppressor genes. For example, RB has been shown to interact with the class of D-type cyclins, as well as a number of transcription factors including E2F, ELF-1, and MYC (Ewen *et al.*, 1993; Dowdy *et al.*, 1993; Kato *et al.*, 1993; Wang *et al.*, 1993; Nevins, 1992; Rustgi *et al.*, 1991). Whether RB will complex with any of these transcription factors depends

on the phosphorylation status of RB, which is believed to be mediated by cyclins D2,D3, E, CDK2, and CDK4 during the G1 phase of the cell cycle (Ewen, 1994). P53 has been shown to function as a transcriptional activator of genes involved in cell cycle progression, such as $p21^{WAF1/CIP1}$, GADD45, and MDM2 (Kastan *et al.*, 1995). $p21^{WAF1/CIP1}$ has been proposed to mediate cell cycle arrest by inhibiting the activity of G1 cyclin-dependent kinases (Harper *et al.*, 1993), while MDM2 appears to function as an inhibitor of P53 activity (Momand *et al.*, 1992). Experiments by Smith *et al.* (1994) have suggested that GADD45 may be involved in DNA repair.

1.5 OSTEOPONTIN

1.5.1 Introduction

One gene that is responsive to induction by *ras* and which frequently has been associated with transformation is osteopontin (OPN). OPN is a secreted phosphoprotein that can bind calcium (Ca^{2+}) and that can bind to cells via interaction of its GRGDS (Gly-Arg-Gly-Asp-Ser) cell binding site with integrin receptors located on the surface of cells (Chen *et al.*, 1992; Miyauchi *et al.*, 1991; Ross *et al.*, 1993; Liaw *et al.*, 1995). OPN has been postulated to play a role in Ca^{2+}-dependent processes, and to function in cell adhesion and signal transduction. The protein is expressed in many tissues and cells including: bone, kidney, dentin, placenta, uterus, decidua, brain, activated T-lymphocytes, activated macrophages, activated natural killer (NK) cells, luminal epithelial cells, smooth muscle cells, endothelial cells after vascular injury, and neurosensory cells of the inner ear (reviewed by Brown *et al.*, 1992; Denhardt and Guo, 1993; Patarca *et al.*, 1993). OPN is also present in many body fluids including milk, plasma, bile and urine (reviewed by Denhardt and Guo, 1993). Expression of OPN is tightly regulated and can be modulated by hormones, growth factors, and tumor promoters (Craig and Denhardt, 1991). In addition to its expression under normal physiological conditions, OPN has also been reported to be associated with a number of pathological conditions including bacterial infection, autoimmune disease, glomerulonephritis, atherosclerosis, and cancer (Patarca *et al.*, 1993; Pichler *et al.*, 1994; Giachelli *et al.*, 1995; Denhardt and Guo, 1993). OPN was characterized

independently by several groups and hence is also known as: **2ar** (Smith and Denhardt, 1987; Craig *et al.*, 1988), **early T-lymphocyte activation-1 (Eta-1) protein** (Patarca *et al.*, 1989), **44 kD bone phosphoprotein** (Prince *et al.*, 1987; Butler, 1989), **phosphoprotein 69 (pp69)** (Chackalaparampil *et al.*, 1985), **bone sialoprotein 1** (Fisher *et al.*, 1987; Kiefer *et al.*, 1989; Young *et al.*, 1990), **secreted phosphoprotein-1 (SPP-1)** (Senger *et al.*, 1989a), **uropontin** (Shiraga *et al.*, 1992; Worcester *et al.*, 1992), and **transformation-associated phosphoprotein** (Senger *et al.*, 1983; 1985).

1.5.2 The *opn* Gene and cDNA Sequence

To date, the *opn* cDNA sequences from seven species have been cloned and characterized; these include: rat, mouse, pig, human, bovine, chicken, and rabbit (Oldberg *et al.*, 1986; Craig *et al.*, 1989; Patarca *et al.*, 1989; Miyazaki *et al.*, 1989; Wrana *et al.*, 1989; Kiefer *et al.*, 1989; Young *et al.*, 1990; Kerr *et al.*, 1991; Crivello and Delvin, 1992; Moore *et al.*, 1991; Castagnola *et al.*, 1991; Tezuka *et al.*, 1992). The *opn* gene structure from three of these species, mouse, chicken, and human have been reported (Miyazaki *et al.*, 1990; Craig and Denhardt, 1991; Rafidi *et al.*, 1994; Hijiya *et al.*, 1994), and sequence analysis and characterization of the *opn* promoter region have been reported for mouse, pig, chicken, and human (Miyazaki *et al.*, 1990; Craig and Denhardt, 1991; Zhang *et al.*, 1992; Rafidi *et al.*, 1994; Hijiya *et al.*, 1994).

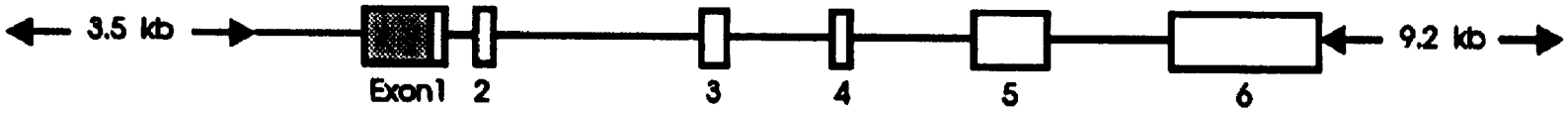
The 5′ untranslated portion of the mouse *opn* gene including a substantial part of the promoter/enhancer region has been independently characterized by Miyazaki *et al.* (1990) and Craig and Denhardt (1991; Craig, 1989)) (see Figure 1.5.2). However, these two groups presented conflicting results regarding the location of the promoter, the location of the transcriptional start site, and the location of the first exon. Work performed as part of this thesis and published by Behrend *et al.* (1993) has resolved this dispute over the structure of the mouse *opn* gene. This work is discussed in detail in chapter 4 of this thesis.

Extensive homology exists between human chromosome 4, mouse chromosome 5, and porcine chromosome 8 with regard to the genetic markers that are located on these chromosomes (Young *et al.*, 1990; Ellegren *et al.*, 1993). Indeed, *opn* has been loca-

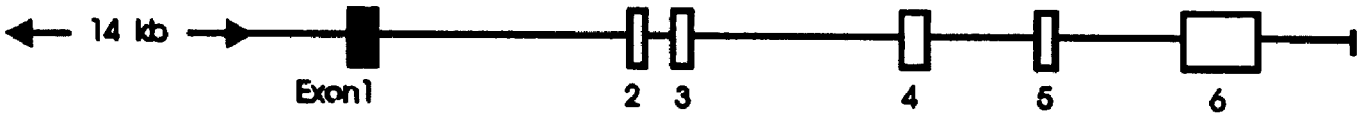
Figure 1.5.2. Maps of the two mouse *opn* genomic clones reported by Miyazaki *et al.* (1990) and Craig and Denhardt (1991). Genomic clone (A) was sequenced (Miyazaki *et al.*, 1990). Exons 1, 3, and 4 of genomic clone (B) were sequenced, whereas exons 2, 5, and 6 were initially mapped by Southern blot analysis (Craig, 1989) and were subsequently localized more accurately from the sequence data of Miyazaki *et al.* (1990). The genomic clones were aligned by restriction enzyme sites (not shown here) and sequence data (for which there were no discrepancies). The *shaded* box indicates the 5′ end of exon 1 as assigned by Miyazaki *et al.* (1990), which corresponds to the 5′ end of an *opn* cDNA reported by Patarca *et al.* (1989). The *blackened* box indicates exon 1 as reported by Craig and Denhardt (1991), which corresponds to the 5′ ends of *opn* mouse and rat cDNAs reported by Craig *et al.* (1989) and Oldberg *et al.* (1986), respectively. (Genomic clones are not drawn to scale; Figure modified from Behrend *et al.*, 1993).

Mouse opn Genomic Clones

(A) Miyazaki et al. (1990)



(B) Craig and Denhardt (1991)



(7)

lized to mouse chromosome 5 (Patarca *et al.*, 1989; Fet *et al.*, 1989; Patarca *et al.*, 1993), to human chromosome 4q13 (Young *et al.*, 1990), and to the porcine chromosome 8 (Ellegren *et al.*, 1993).

It has been shown that in the mouse and human genomes *opn* exists as a single-copy gene (Craig, 1989; Craig *et al.*, 1989; Fet *et al.*, 1989; Miyazaki *et al.*, 1990; Young *et al.*, 1990). However, Crivello and Delvin (1992) have argued that two related but distinct *opn* genes exist in the bovine genome. Crivello and Delvin (1992) isolated an *opn* cDNA from bovine kidney, termed *opn-k*. Apparently, the tissue distribution and nucleotide sequence of bovine *opn-k* were different enough from those of the bovine bone *opn* cDNA (Kerr *et al.*, 1991) and other reported *opn* cDNA species, that Crivello and Delvin (1992) designated *opn-k* as a highly related, but distinct gene.

Evidence for the differential splicing of *opn* RNA has come from a number of different sources. Young *et al.* (1990) isolated a human *opn* cDNA clone that differed from the human *opn* cDNA clone reported by Kiefer *et al.* (1989) by the absence of 42 nucleotides. Northern blot analysis revealed that both *opn* mRNA species were present in human bone and decidua cells (Young *et al.*, 1990). Since *opn* is a single-copy gene, these findings suggested that the two human *opn* mRNA species may have been generated by differential RNA splicing. The presence of a splice consensus sequence (AG) at the 3′ end of this 42-nucleotide insert/gap indicates that splicing may be possible at this site (Young *et al.*, 1990).

Singh *et al.* (1992) isolated two cDNA clones from Kirsten sarcoma virus-transformed normal rat kidney (KNRK) cells and ROS 17/2.8 rat osteosarcoma cells, that differed by a 52-nucleotide-long insert in the 5′non-coding region of the KNRK cell-derived cDNA. Putative intron splice junctions around the insert, the location of the insert 15 nucleotides upstream of the translational start site, and the presence of the insert in the genomic DNA of both NRK and ROS cells amongst other factors, prov ,ed strong evidence for differential, cell type-specific processing of the *opn* transcripts.

Most recently, Saitoh *et al.* (1995) isolated and sequenced three *opn* splice variants from human glioma cell lines, *opn-a*, *opn-b*, and *opn-c*. *Opn-a* and *opn-b* were identical to the two human *opn* mRNA species identified by Kiefer *et al.* (1989) and

Young *et al.* (1990), respectively. *Opn-c* (864 bp) was a novel species of *opn* cDNA that lacked an 81-nucleotide sequence, present in the 5′ region of both *opn-a* and *opn-b* variants (Saitoh *et al.*, 1995).

The significance of different splicing variants for *opn* is not known at present. However, splicing variants may play a role in the tissue-specific expression and function of OPN.

1.5.3 Regulation of *opn* Expression

Expression of *opn* can be modulated by a variety of agents, including hormones, growth factors, tumor promoters and oncogenes. Mouse *opn* mRNA was initially identified based on its inducibility by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in JB6 mouse epidermal cells (Smith and Denhardt, 1987), and is also induced by TPA in mouse epidermis *in vivo* (Craig *et al.*, 1989). A number of sequence motifs identified in the *opn* promoter may be involved in mediating the TPA inducibility of cells; these include AP-1 (activator protein-1), AP-2, PEA-3 (polyoma enhancer activator-3), AP-4, and AP-5 (Denhardt and Guo, 1993).

It has long been recognized that expression of *opn* is increased in cells that are transformed, regardless of the transforming agent (Craig *et al.*, 1988; Senger *et al.*, 1989a), and that *opn* expression correlates with the metastatic potential of murine fibroblasts (Craig *et al.*, 1990).

Expression of *opn* is positively regulated by a number of different agents, including: calcitriol (vitamin D_3) (Prince and Butler, 1987), retinoic acid (Kasugai *et al.*, 1991), estrogen and progesterone (Craig and Denhardt, 1991), leukemia inhibitory factor (Noda *et al.*, 1990a), prostaglandin E2 (Nagata *et al.*, 1994), insulin-like growth factor-I (Tanaka *et al.*, 1994), interleukin-1α (Jin *et al.*, 1990), epidermal growth factor (Laverdure *et al.* (1987); and basic fibroblast growth factor (Rodan *et al.*, 1989). Furthermore, expression of *opn* is stimulated in T-lymphocytes that have been activated by concanavalin A or bacterial infection, and in macrophages treated with lipopolysaccharide or lymphokines (Patarca *et al.*, 1989; Miyazaki *et al.*, 1990). Although Noda *et al.* (1988) and Kasugai *et al.* (1991) reported that transforming growth

factor beta (TGF-β) increased expression of *opn* in rat osteosarcoma cells, others reported the activity of TGF-β to be inhibitory on *opn* expression (Laverdure *et al.*, 1987; Zhou *et al.*, 1993; Harris *et al.*, 1994; Staal *et al.*, 1994). Similarly, Yoon *et al.* (1987) reported that dexamethasone negatively regulates *opn* expression in bone, whereas Quarto *et al.* (1995) and Rickard *et al.* (1994) observed increased expression of *opn* in osteoprogenitor cells in response to dexamethasone. Expression of *opn* is inhibited in osteoblast-like cells by parathyroid hormone (Noda and Rodan, 1989b) and in osteoclasts by calcitonin (Kaji *et al.*, 1994).

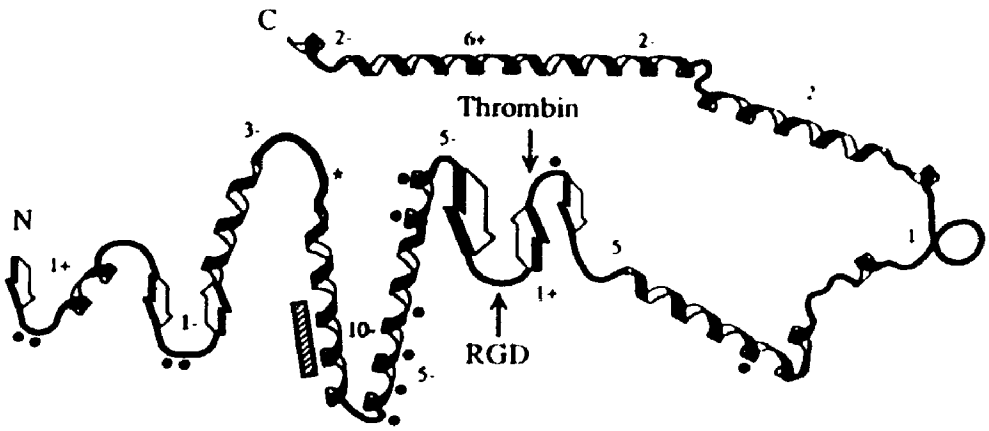
Analysis of the mouse *opn* promoter region for transcription factor recognition sequences has revealed many potential transcription factor binding sites (Denhardt and Guo, 1993). Whether all or only some of them are functional remains to be determined. However, the presence of multiple potential regulatory sites hints at the complexity of *opn* gene regulation.

1.5.4 The OPN Protein: Primary Sequence Motifs

Mouse OPN is a secreted phosphoprotein that is composed of 294 amino acids (Craig *et al.*, 1989). Mouse OPN is very similar to rat OPN (317 amino acids) in both amino acid level identity (84%) and nucleotide sequence identity (87%) (Craig *et al.*, 1989; Oldberg *et al.*, 1986). OPN proteins from all species identified contain a number of primary sequence motifs, including a cell binding sequence (Gly-Arg-Gly-Asp-Ser (GRGDS)), a polyaspartic acid motif, three potential thrombin cleavage sites, a potential Ca^{2+} binding site, and two heparan binding domains (Oldberg *et al.*, 1986; Craig *et al.*, 1989; Patarca *et al.*, 1989; Kiefer *et al.*, 1989; Wrana *et al.*, 1989; Young *et al.*, 1990; Kerr *et al.*, 1991) (see Figure 1.5.4; redrawn from Denhardt and Guo (1993)).

OPN is a member of the group of acidic, non-collagenous glycoproteins of mineralized tissues which include osteocalcin, osteonectin, bone sialoprotein (BSP), matrix Gla protein, bone acidic glycoprotein-75 (BAG-75), and dentin sialoprotein (DSP) (Heinegård and Oldberg, 1989; Butler, 1991; Roach, 1994). Almost half of the amino acid content of OPN is composed of serine, aspartate, and glutamate residues, giving the protein backbone an overall net negative charge (see Figure 1.5.4; redrawn from Den-

Figure 1.5.4. Cartoon representation of the OPN protein. Regions of predicted α-helices and β-sheet structure are indicated by *helices* and *arrows*, respectively. The *shaded* box and *solid circles* indicate the stretch of aspartic acid residues and putative sites of serine/threonine phosphorylation, respectively. The conserved RGD site and thrombin cleavage site are marked. The *star* indicates an *N*-linked glycosylation site. The negative and positive numbers along the chain indicate at 20-residue intervals the net charge in that region of the polypeptide backbone at neutral pH. (Figure modified from Denhardt and Guo, 1993).


C
2-
6+
2-
Thrombin
3-
5-
N
1+
1-
10-
5-
RGD
1+
5
1

hardt and Guo (1993)). In addition, there is a stretch of 7 to 10 consecutive aspartic acid residues, depending on the species, which is highly conserved in all OPN proteins regardless of the tissue of origin or the species (Oldberg *et al.*, 1986; Craig *et al.*, 1989; Fisher *et al.*, 1990; Zhang *et al.*, 1990; Crivello and Delvin, 1992). It has been postulated that this stretch may constitute a potential hydroxyapatite (HA) binding region (Butler, 1989). Recent work by Boskey *et al.* (1993), Hunter and Goldberg (1993), and Hunter *et al.* (1994) support this notion. Bovine OPN (Boskey *et al.*, 1993) as well as OPN protein from adult porcine calvarial bone and the synthetic polypeptide poly(aspartic acid) (Hunter *et al.*, 1994) caused inhibition of HA formation, while control proteins as well as the control polypeptide poly(glutamic acid) had little or no inhibitory effect on HA formation. Phosphorylation and carboxylation of OPN are essential for the protein's HA inhibitory activity, and the poly(aspartic acid) motif of OPN may be involved in mediating this process (Boskey *et al.*, 1993; Hunter and Goldberg, 1993; Hunter *et al.*, 1994).

OPN protein also contains a Gly-Arg-Gly-Asp-Ser (GRGDS) cell adhesion site, which is common in a number of proteins that bind to cell surface receptors called integrins (Ruoslahti and Pierschbacher. 1987). The GRGDS cell binding domain is conserved in all OPN species identified (Oldberg *et al.*, 1986; Craig *et al.*, 1989; Patarca *et al.*, 1989; Miyazaki *et al.*, 1989; Wrana *et al.*, 1989; Kiefer *et al.*, 1989; Young *et al.*, 1990; Kerr *et al.*, 1991; Crivello and Delvin, 1992; Moore *et al.*, 1991; Castagnola *et al.*, 1991; Tezuka *et al.*, 1992). Both native and recombinant OPN proteins have been shown to mediate adhesion of OPN to many different cell types, including osteoblasts, osteoclasts, gingival fibroblasts, cultured smooth muscle and endothelial cells, and many transformed cell lines (Oldberg *et al.*, 1986; Somerman *et al.*, 1987; 1988; 1989; Liaw *et al.*, 1994; Chambers *et al.*, 1993; Xuan *et al.*, 1994). Adhesion of cells to OPN can be blocked by RGD-containing peptides, a monoclonal antibody directed against the OPN RGD region, or by site-directed mutagenesis of the OPN RGD sequence, suggesting that adhesion of OPN to cells is specific and mediated via the RGD sequence (Oldberg *et al.*, 1986; Chambers *et al.*, 1993; Bautista *et al.*, 1994; Xuan *et al.*, 1995). However, van Dijk *et al.* (1993) argue that non-RGD-mediated interactions are also involved in adhe-

sion of rat OPN to human gingival fibroblasts, since a 28 kD peptide of OPN, an endoproteinase Arg-C digestion product lacking the RGD sequence, was capable of promoting cell attachment to a similar degree as native OPN. Similar results were observed by Katagiri *et al.* (1996) who have identified a cell line, B16-BL6 melanoma cells, that can bind to OPN via RGD- and α_v integrin-independent interactions. This observation that some cells can interact with OPN via non-RGD binding sites is further supported by the recent finding that OPN serves as a ligand for the CD44 receptor in hematopoietic cells (Weber *et al.*, 1996).

Binding activity of OPN is not limited to cells. The protein has been reported to associate with fibronectin (Nemir *et al.*, 1989), osteocalcin (Ritter *et al.* 1992), and type I collagen (Chen *et al.*, 1992). The association between OPN and fibronectin is mediated via covalent cross-linkings between OPN and fibronectin, and is catalyzed by transglutaminase (TG) (Prince *et al.*, 1991; Beninati *et al.*, 1994). Two TG-reactive glutamine residues have been identified in bovine OPN and are conserved in all known OPN sequences (Sorensen *et al.*, 1994). Beninati *et al.* (1994) have further shown that TG-mediated cross-linking between OPN and fibronectin occurs *in vivo*, suggesting TG-mediated cross-linking as one mechanism by which OPN may be incorporated into biological matrices such as bone or urinary stones.

Another notable feature of the OPN protein are the three potential thrombin cleavage sites at the carboxyl-side of residues Arg_{144}, Arg_{153}, and Arg_{157} (Craig *et al.*, 1989). A major thrombin cleavage site which is located approximately 6 amino acids from the RGD cell binding site (toward the C-terminus of the protein) has been described by Senger *et al.* (1988; 1989b). A minor thrombin cleavage site was reported by Ashkar *et al.* (1993b) to be located 4 amino acids from the major cleavage site (10 amino acids away from the RGD sequence). Enzymatic cleavage of OPN by thrombin may represent an important regulator of OPN function, since thrombin-cleaved OPN has been reported to no longer support adhesion of cells to the substratum (Xuan *et al.*, 1994). In contrast, Senger *et al.* (1994) have reported that cleavage of OPN by thrombin greatly increased the cell attachment and spreading capabilities of OPN for a number of cell lines. Although the function of thrombin-cleaved OPN is controversial at present, both reports

demonstrate the importance of thrombin cleavage in regulating the function of OPN.

1.5.5 The OPN Protein: Post-translational Modifications

OPN protein is subject to a variety of post-translational modifications. Rat bone OPN is phosphorylated at 12 serine residues and 1 threonine residue, and contains 10 sialic acid residues, as well as 1 *N*-linked and 5 to 6 *O*-linked oligosaccharides (Prince *et al.*, 1987). Sulfated species of OPN have been described by Nagata *et al.* (1989). Frequently, more than one species of OPN protein have been reported on SDS-polyacrylamide gels (44 and 55 kD, or 62 and 69 kD), differing either in the amount of post-translational modification or possibly representing differentially spliced forms of OPN (Nagata *et al.*, 1989; Kubota *et al.*, 1989). By sedimentation equilibrium centrifugation, glycosylated, phosphorylated OPN protein has a relative molecular weight (M_r) of 41,500 (Prince *et al.*, 1987). However, the apparent molecular weight of OPN based on electrophoretic mobility in SDS-polyacrylamide gels varies widely (from 44 to 75 kD) depending on the acrylamide concentration and the extent of cross-linking, and has been attributed to the high negative charge imposed on the protein by the acidic amino acids and the high degree of phosphorylation.

OPN protein is highly phosphorylated, and candidate protein kinases for the phosphorylation of OPN are casein kinase II and cAMP-dependent protein kinase, which have been shown to phosphorylate recombinant mouse OPN expressed in *Escherichia coli (E. coli) in vitro* (Ashkar *et al.*, 1993b). Furthermore, recombinant mouse OPN protein from *E. coli* has been reported to be capable of autophosphorylation on tyrosine residues (Ashkar *et al.*, 1993a), although this has not yet been demonstrated for OPN produced by mammalian cells. Amino acid sequence analysis of bovine milk OPN has revealed phosphorylation on 27 serine and 1 threonine residue (Sorensen *et al.*, 1995). Twenty-five of the phosphoserines and 1 phosphothreonine are located in a sequence motif (Ser/Thr-X-Glu/Ser(P)/Asp), suggesting that phosphorylation is catalyzed by the mammary gland casein kinase, while the two remaining phosphoserines are located in a sequence motif (Ser-X-X-Glu/Ser(P)) which is a recognition sequence for casein kinase II. The serine in the cell binding sequence Arg-Gly-Asp-Ser of OPN does not appear to

be phosphorylated.

OPN can be partially dephosphorylated by tartrate-resistant acid phosphatase (TRAP) of skeletal osteoclasts, after which the protein can no longer support binding of osteoclasts to glass plates (Ek-Rylander *et al.*, 1994). This finding is in contrast to the observations reported by Xuan *et al.* (1994; 1995) where recombinant OPN protein made in *E. coli* did promote cell adhesion.

Several studies have reported the existence of non-phosphorylated forms of OPN (Nemir *et al.*, 1989; Chang and Prince, 1991). Normal rat kidney (NRK) cells secrete both phosphorylated (pp62 and pp69) and non-phosphorylated (np69) forms of OPN (Nemir *et al.*, 1989). These different forms of OPN may play different functional roles, since phosphorylated OPN (pp69) was capable of binding to cell surface-associated fibronectin, while non-phosphorylated OPN (np69) could form an immunoprecipitate with soluble fibronectin (Nemir *et al.*, 1989). In addition, Singh *et al.* (1990a) have shown that np69, but not pp69, contains *N*-linked carbohydrates, illustrating that physiological properties of different OPN species are regulated by post-translational modifications. Chang and Prince (1991) have shown that calcitriol treatment of a clonal isolate of mouse JB6 epidermal cells (C141.5a) induced the synthesis and secretion of non-phosphorylated OPN, but did not transform the cells. Treatment with the tumor promoter TPA, on the other hand, induced synthesis and secretion of phosphorylated OPN and resulted in tumorigenic transformation of the cells. These observations suggested an important role for the phosphorylation of OPN in the transformation of these cells. Treatment of JB6 C141.5a cells with calcitriol and TPA resulted in the production of a more highly phosphorylated form of OPN and enhanced TPA-induced transformation several-fold, suggesting a coordinate regulation between the signaling pathways for calcitriol and TPA in JB6 C141.5a cells and further implicating expression of phosphorylated OPN in tumorigenesis (Chang and Prince, 1993).

1.5.6 The Role of OPN in Cell Signaling

OPN has been shown to mediate attachment and cell spreading of osteosarcoma cells, periodontal ligament cells, and fibroblasts likely through interaction with the vitro-

nectin receptor $\alpha_v\beta_3$ (Somerman *et al.*, 1987; 1988; 1989a; 1989b; Reinholt *et al.*, 1990; Smith and Cheresh, 1990; Flores *et al.*, 1992; Helfrich *et al.*, 1992; Ross *et al.*, 1993; Chambers *et al.*, 1993). Interaction between OPN and the cell is RGD-dependent and inhibited by RGD-containing peptides and a monoclonal antibody directed against the OPN RGD region (Flores *et al.*, 1992; Chambers *et al.*, 1993; Bautista *et al.*, 1994). Many lines of research have now established that integrins are capable of transmitting signals both into and out of the cell (reviewed by Hynes, 1992; Schwartz, 1993; Juliano, 1994; Clark and Brugge, 1995). Some of the integrin-dependent signaling events that have been described include: protein phosphorylation of kinases such as focal adhesion kinase (FAK); the Src family of tyrosine kinases; and growth factor receptor tyrosine kinases; phospholipid turn-over; intracellular calcium regulation; the RAS-MAP kinase pathway; G protein-coupled receptor pathways; and cytokine and immune receptors pathways (reviewed by Clark and Brugge, 1995). Evidence is accumulating for a role for OPN in signaling via interaction with $\alpha_v\beta_3$. Sauk *et al.* (1990) have reported enhanced expression of heat shock proteins HSP70 and HSP47 as a result of persistent spreading of cells on OPN. Chambers *et al.* (1993) observed that *ras*-transformed NIH 3T3 cells adhered to and spread on OPN-coated surfaces, while control NIH 3T3 cells adhered but spread poorly on OPN-coated surfaces. An analysis of the kinetics of adhesion revealed that adhesion was maximal at 30-60 minutes with decreased adhesion thereafter, suggesting that the interaction between OPN and the cell may involve signaling rather than simple adhesion (Chambers *et al.*, 1993). While Miyauchi *et al.* (1991; 1993) reported a calmodulin-dependent reduction in cytosolic calcium as a result of OPN binding to $\alpha_v\beta_3$ on osteoclasts, Zimolo *et al.* (1994) observed a transient increase in intracellular calcium in osteoblast-like cells treated with OPN. Yue *et al.* (1994) observed increased migration of aortic smooth muscle cells in response to OPN, suggesting that OPN may be involved in the cells response to vascular injury. Clyman *et al.* (1992) and Liaw *et al.* (1995) have demonstrated that the migratory effect of OPN on cells is mediated only by $\alpha_v\beta_3$, and not $\alpha_v\beta_1$ or $\alpha_v\beta_5$, which have been identified as novel OPN receptors that support adhesion of cells to OPN. More indirect evidence for a role of OPN in cell signaling comes from studies implicating $\alpha_v\beta_3$ in signaling, such

as the concomitant increases in invasiveness and secretion of matrix metalloproteinase as a result of perturbation of the $\alpha_v\beta_3$ receptor (Seftor *et al.*, 1992; 1993). Another clue for the role of OPN in signaling comes from the studies of Hwang *et al.* (1994a). OPN has been shown to inhibit nitric oxide (NO) production by suppressing induction of the inducible nitric oxide synthase (iNOS) mRNA in kidney epithelial cells treated with gamma-interferon and lipopolysaccharide (LPS). NO is an important signaling molecule and a regulator of many cellular functions, including renal absorption and vascular tone. By controlling NO production, OPN may function as a regulator of NO and NO-mediated processes.

1.5.7 The Role of OPN in Bone

In bone, OPN has been shown to be a prominent component of the mineralized bone extracellular matrix (Franzén and Heinegård, 1985; Prince *et al.*, 1987; Fisher *et al.*, 1987). A large collection of data suggest multiple functions for OPN in the development and maintenance of this tissue, including bone formation and mineralization, and a role in promoting osteoclast attachment and bone resorption.

Two major cell types involved in bone remodelling are osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) (Butler, 1989). OPN has long been known to be expressed by osteoblasts, with low levels of expression being detected in preosteoblasts prior to mineralization, and markedly elevated levels in mature osteoblasts (Mark *et al.*, 1987; 1988; Hultenby *et al.*, 1991; Chen *et al.*, 1993; Lian *et al.*, 1993). Merry *et al.* (1993) reported expression of OPN by osteoclasts. The expression of OPN by preosteoblasts early in bone development is consistent with a role for OPN in bone formation (Chen *et al.*, 1993; Yao *et al.*, 1994). Ultrastructural immuno-localization studies have localized OPN to bone surfaces (lamina limitans) and cement lines, and to the mineralization front of newly formed bone, suggesting a role for OPN in attachment of bone cells and in mineralization (Chen *et al.*, 1991; Hultenby *et al.*, 1991; McKee *et al.*, 1992; Chen *et al.*, 1993; McKee *et al.*, 1993; Chen *et al.*, 1994). The role OPN plays during mineralization appears to be regulatory as opposed to initiating mineralization, since OPN does not promote nucleation of hydroxyapatite (HA), but

rather functions as an inhibitor of hydroxyapatite formation when fully phosphorylated (Boskey *et al.*, 1993; Hunter and Goldberg, 1993; Hunter *et al.*, 1994). High expression of OPN by osteoblasts later in bone development, together with high expression at sites of bone remodelling and osteoclast activity, implicate OPN in bone resorption mediated by osteoclasts (Mark *et al.*, 1987; 1988; Reinholt *et al.*, 1990; Hultenby *et al.*, 1991; Chen *et al.*, 1993; Arai *et al.*, 1993; Hultenby *et al.*, 1994; Takano-Yamamoto *et al.*, 1994; Ikeda *et al.*, 1992; 1994). Flores *et al.* (1992) have shown that osteoclasts can bind OPN via an RGD-mediated interaction, and that OPN concentrations are enhanced in bone opposite the clear zone of the osteoclast, which is a region of adherence between the osteoclast and the bone surface where the localized acidic conditions promote resorption of the bone matrix. OPN in the mineralized bone matrix may function to attract osteoclasts to the site of resorption (Boskey, 1995), function as an anchor to attach osteoclasts to bone surfaces (Reinholt *et al.*, 1990; Hultenby *et al.*, 1993; 1994; Heinegård *et al.*, 1995), or promote the bone-resorbing activity of osteoclasts (Jin *et al.*, 1990). Any or all of these potential functions for OPN in the mineralized bone matrix are likely to be mediated via $\alpha_v\beta_3$ (Helfrich *et al.*, 1992; Ross *et al.*, 1993). Heinegård *et al.* (1995) propose that following bone resorption, dephosphorylation of OPN by tartrate-resistant acid phosphatase (TRAP) may mediate detachment of the osteoclast from the resorption site, since OPN dephosphorylated by TRAP could no longer support binding of osteoclasts *in vitro* (Ek-Rylander *et al.*, 1994).

Two different species of phosphorylated OPN have been identified in cultured rat bone cells, a highly phosphorylated 44 kD form and a less phosphorylated 55 kD form (Nagata *et al.*, 1989; Kubota *et al.*, 1989). The 55 kD species is produced by preosteoblastic cells and correlates with the formation of a cement layer upon which mineralized bone tissue is formed, while the 44 kD species is produced by mature osteoblasts and associates rapidly with hydroxyapatite where it may regulate hydroxyapatite crystal growth (Nagata *et al.*, 1991; Kasugai *et al.*, 1992; Sodek *et al.*, 1995). Expression of OPN is stimulated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), vitamin D3, retinoic acid, and mechanical stress (Prince and Butler, 1987; Noda and Rodan, 1989a;

Kasugai *et al.*, 1991; Kubota *et al.*, 1993; Harter *et al.*, 1995). PDGF, EGF, and TGF-β are growth factors that promote bone formation, while vitamin D3 and retinoic acid are osteotropic hormones that promote bone resorption, consistent with a potential function for OPN in both processes.

1.5.8 The Role of OPN in Kidney and Urine

OPN is also found in the kidneys, particularly in the long loop of Henle and the distal convoluted tubule of nephrons (Lopez *et al.*, 1993). Hwang *et al.* (1994a) have shown that OPN can inhibit production of nitric oxide (NO), a short-lived gaseous reactive metabolite that is involved in the regulation of renal absorption and vascular tone. Furthermore, Hwang *et al.* (1994b) demonstrated that expression of both *opn* mRNA and *cNOS* (constitutive nitric oxide synthase) mRNA from kidney proximal tubule epithelial (PTE) cells increased with the age of the kidney donor. PTE cells from young, but not old, kidneys showed increased *opn* mRNA expression and decreased *cNOS* mRNA expression after exposure to hypoxia-reoxygenation conditions (Hwang *et al.*, 1994b). Presumably PTE cells from old kidneys have a diminished capacity to increase *opn* expression and decrease NO production, which renders them more susceptible to oxidant injury.

OPN protein is also present in urine where it is believed to play a role in inhibiting urinary stone formation (Shiraga *et al.*, 1992; Worcester *et al.*, 1992; Worcester, 1994). OPN is a component of calcium oxalate crystals, and presumably inhibits stone formation by disrupting the crystallization process (Shiraga *et al.*, 1992; Worcester *et al.*, 1992; Kohri *et al.*, 1993). This finding is consistent with the reports by Hunter and Goldberg (1993) and Hunter *et al.* (1994), who have shown that OPN inhibits hydroxyapatite (HA) formation. Recently, Bautista *et al.* (1996) determined the levels of OPN protein in urine of both patients with kidney stones and normal individuals. The levels of OPN protein in urine did not vary between normal individuals and patients, ranging from 0.01 to 2.7 μg/ml for all individuals tested. However, the urine of a significant portion of kidney stone patients contained aberrant OPN species ($\leq$40 kD versus 55-66 kD for normal urine-derived OPN) (Bautista *et al.*, 1996). In

addition, Bautista *et al.* (1996) detected serine proteases inhibitable with phenylmethylsulfonyl fluoride in urine samples containing aberrant OPN, indicating that these proteases contributed to the proteolytic cleavage of urine OPN.

A number of reports implicated OPN in the development of nephritis. In an angiotensin II-induced model of tubulointerstitial nephritis, OPN protein and mRNA are increased in select regions of the kidney, which correspond to sites of monocyte/macrophage accumulation (Giachelli *et al.*, 1994). Pichler *et al.* (1994) have demonstrated that OPN expression is up-regulated in proximal and distal tubules in three experimental models of glomerulonephritis. Expression of OPN precedes histological evidence of tubular injury, correlates with sites of monocyte/macrophage accumulation and tubular injury, and correlates with the severity of tubular injury (Pichler *et al.*, 1994). Similar increases in OPN expression were also reported for another model of glomerulonephritis (Couser and Johnson, 1994), raising the possibility that OPN plays a role in the pathogenesis of the tubular injury that accompanies this disease.

1.5.9 The Role of OPN in the Vasculature

OPN also appears to play a role in the vascular system and in vascular disease (Giachelli *et al.*, 1995). Low levels of OPN are produced by uninjured arterial smooth muscle cells (SMC) (Giachelli *et al.*, 1993). Expression of OPN is enhanced in SMC in response to vascular injury, in proliferating SMC, and in SMC grown *in vitro* and stimulated with basic fibroblast growth factor, transforming growth factor beta, or angiotensin II (Gadeau *et al.*, 1993; Giachelli *et al.*, 1993). OPN has been detected in a growing number of cardiovascular pathologies, where it is most often associated with calcified plaques (Giachelli *et al.*, 1993; Fitzpatrick *et al.*, 1994; Shanahan *et al.*, 1994; Giachelli *et al.*, 1995). The cells that produce OPN which is found in the calcified plaques have been identified as macrophages, SMC, and endothelial cells (O'Brien *et al.*, 1994). Liaw *et al.* (1994) have shown that OPN promotes adhesion of both cultured aortic endothelial cells and aortic SMC, and that this interaction is blocked by an anti-$\alpha_v\beta_3$ antibody. Integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$ also support adhesion of SMC to OPN, but migration of SMC in response to OPN is mediated by $\alpha_v\beta_3$ (Liaw *et al.*, 1995; Yue *et*

al., 1994). Most recently, Liaw *et al.* (1995) have shown that proliferating arterial endothelial cells express increased levels of *opn* mRNA and OPN protein as well as increased levels of β_3 integrin mRNA and protein in response to vascular injury. *In vitro*, OPN promoted adhesion and migration of aortic endothelial cells. These results provide some indication of the complex functions OPN may have in vascular homeostasis, regeneration, and disease development.

1.5.10 The Role of OPN in Other Tissues

OPN is detected in the sensory epithelium of the inner ear, where it has been postulated to be involved in development, calcium regulation, mineralization, and regulation of nitric oxide production by neurons (Davis *et al.*, 1995). Senger *et al.* (1989b) have identified OPN in human milk at concentrations of 3-10 μg/ml. The function of OPN in milk is not known, but may include protection against bacterial or viral infection (Patarca *et al.*, 1989; Patarca *et al.*, 1993; Denhardt and Guo, 1993). OPN is detected in granulated metrial gland cells of the decidua and placenta of pregnant mice, and expression is regulated both spatially and temporally (Waterhouse *et al.*, 1992). OPN is found in the ovaries, and the skin and ventral fatty tissue of pregnant and lactating mice (Craig and Denhardt, 1991). Recently, OPN has also been identified in rat testis, epididymis, and spermatozoa (Siiteri *et al.*, 1995). Brown *et al.* (1992) have localized OPN to the luminal surface of epithelial cells in the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung bronchi, and in mammary, salivary, and sweat glands. OPN has also been detected in bile. OPN secreted by luminal epithelial cells may bind to integrins on the luminal surfaces, and play an important role in the communication of luminal epithelial cells with the external environment (Brown *et al.*, 1992). Ultrastructural immunogold localization of OPN in gallbladder epithelial cells has demonstrated that a portion of the secreted OPN protein remains associated with the cell surface and becomes integrated into the filamentous glycocalyx of luminal gallbladder epithelial cells (Hong *et al.*, 1994). Murray *et al.* (1994) have detected OPN in macrophages during repair of myocardial necrosis, and have proposed a role for OPN in wound healing following cardiac tissue injury.

1.5.11 The Role of OPN in Immunity and Infection

OPN also appears to play an as yet poorly defined role in immunity. The efforts to identify genes involved in immunological resistance to bacterial infection led Patarca and colleagues (1989) to discover OPN in T cells and a subset of natural killer (NK) cells. Expression of the *opn* gene is markedly elevated in T lymphocytes shortly after activation of the cells with concanavalin A or bacterial infection, hence the name Eta-1 (early T-lymphocyte activation 1) protein (Patarca *et al.*, 1989). ETA-1 is expressed *in vivo* in response to bacterial infection, and maps to the *Ric* locus on mouse chromosome 5, which confers genetic resistance to infection by the obligate intracellular bacterium *Rickettsia tsutsugamushi (RT)* and possibly *Listeria monocytogenes* (Patarca *et al.*, 1989; Fet *et al.*, 1989; Patarca *et al.*, 1993). Three structural alleles of this single copy gene have been identified with respect to bacterial resistance in a variety of inbred mouse strains; ETA-1^a, ETA-1^b, and ETA-1^c (Patarca *et al.*, 1989; Patarca *et al.*, 1993). ETA-1^a is associated with resistance to bacterial infection, while ETA-1^b and ETA-1^c abolish early ETA-1 induction in response to *RT* infection and are associated with susceptibility to *Rickettsial* infection.

Patients with Gram-negative sepsis have been shown to contain high levels of circulating OPN in their serum (Senger *et al.*, 1988). The source of the OPN in patients with sepsis are likely to be macrophages. Macrophages can be induced to synthesize and secrete OPN upon stimulation with bacterial lipopolysaccharide (LPS) (Miyazaki *et al.*, 1990). In addition, Singh *et al.* (1990b) have shown that purified OPN can bind specifically to murine macrophages via the GRGDS cell binding domain, and that OPN is chemoattractive for macrophages, resulting in a macrophage-rich infiltrate on subcutaneous injection of OPN into mice. These findings raise the possibility that resistance to Gram-negative bacterial infection by OPN may also be mediated via a macrophage pathway, in which OPN may enhance resistance by assisting macrophages in migrating to sites of infection and/or to express bacteriocidal activity (Singh *et al.*, 1990b; Patarca *et al.*, 1993).

It has been proposed that OPN may also be involved in resistance to infection by flaviviruses, a subgroup of RNA viruses that include yellow fever, St. Louis encephalitis,

and others (Patarca *et al.*, 1993). The basis for this hypothesis is the presence of a gene conferring resistance to flavivirus infection in the C3H/RV mouse strain and absence of the gene in the otherwise congenic C3H/He strain (Goodman and Koprowski, 1962). This pair of mouse strains was found to differ at the locus that confers resistance to *Rickettsial* infection (Jerrells and Osterman, 1981).

Finally, Lampe *et al.* (1991) and Patarca *et al.* (1991) have studied the hypothesis that OPN may play a role in murine autoimmune disease. The MRL/lpr inbred mouse strain spontaneously develops a systemic autoimmune disease that resembles human systemic lupus erythematosus (SLE). One hallmark of systemic autoimmune diseases such as SLE is chronic and sustained polyclonal B-cell activation. Lampe and colleagues (1991) have shown that addition of both recombinant and purified native OPN protein to mixtures of B-cells and macrophages from the autoimmune MRL/lpr strain resulted in an increase in both IgM and IgG antibodies, with the increase being directly proportional to the concentration of OPN in the cultures. Patarca *et al.* (1991) reported that expression of OPN is increased significantly in T cells derived from MRL/lpr mice, but not in T cells from normal mouse strains. These findings suggest a potential role for OPN in the development of murine SLE.

Most recently, Cantor (1995) has reported increased levels of OPN in a murine model of acquired immunodeficiency syndrome. Expression of OPN is dramatically increased in these mice, while expression of other cytokines such as interleukin 2, interleukin 3, interleukin 4, and gamma-interferon are not significantly elevated (Cantor, 1995). The function OPN plays in this disease or any other pathological condition remains to be determined.

1.5.12 The Role of OPN in Cancer

Several independent identifications of OPN have been made on the basis of its association with transformation and malignancy. Senger *et al.* (1979; 1980; 1983; 1985) have identified phosphoproteins in the 58 - 62 kD molecular weight range that are secreted from a variety of transformed mammalian cell lines at increased levels (10-fold or greater) relative to the non-transformed parental cell lines. These phosphoproteins are

secreted from the transformed cell lines regardless of the transforming agent, and are antigenically-related (Senger *et al.*, 1979, 1980, 1983, 1985). A similar phosphoprotein, pp69, was identified by Chackalaparampil *et al.* (1985) in Rous sarcoma virus-transformed rat cells. Independently, Smith and Denhardt (1987) reported the identification of an mRNA species, designated "2ar", in JB6 mouse epidermal cells on the basis of its inducibility by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Craig *et al.* (1988) identified "2ar", the major secreted phosphoprotein of many transformed rodent cell lines as being the mouse homologue of rat osteopontin/ 44 kD bone phosphoprotein. In addition, Craig *et al.* (1989; 1990) demonstrated that OPN is induced by TPA in mouse epidermis *in vivo*, and that its expression correlates with tumor progression in the animal, being lowest in normal epidermis, slightly increased in papillomas, and greatly increased in carcinomas. Expression of OPN also correlates with *ras* mRNA levels and metastatic potential (experimental and spontaneous) in murine fibroblasts (Craig *et al.*, 1990). Further characterization of the tumor-derived phosphoprotein by Senger *et al.* (1989a) confirmed Craig *et al.'s* (1988) finding that the tumor-derived secreted phosphoprotein is identical to bone-derived rat OPN. In addition, Senger *et al.* (1988) showed that expression of OPN was increased in the serum of 10 out of 13 patients with disseminated carcinoma, relative to normal controls.

Additional support for OPN's involvement in transformation and tumorigenesis is given by the observation that expression of OPN can be induced by transformation with oncogenes. Craig *et al.* (1988) have shown that OPN protein and cytoplasmic mRNA levels were dramatically increased in NIH 3T3 cells transformed with the human bladder carcinoma T24 H-*ras* oncogene. Both OPN protein and mRNA levels were increased, particularly when the H-*ras*-transformed cells were selected for enhanced metastatic ability (Chambers *et al.*, 1992b). Steady-state levels of OPN were significantly increased in v-*ras*-transformed MC3T3 mouse osteoblasts relative to control cells (Nose *et al.*, 1990). Furthermore, OPN levels were greatly elevated in chondrocytes which constitutively expressed v-*myc* (Castagnola *et al.*, 1991).

Ongoing research into the role of OPN in malignancy has revealed some interesting discoveries. Chang and Prince (1993) have shown that concurrent treatment

of JB6 mouse epidermal cells with calcitriol and TPA increased expression of OPN and anchorage-independent growth. Most notable was the observation that a more highly phosphorylated species of OPN was produced, suggesting that the phosphorylation status of OPN may be important in its contribution to tumorigenicity.

Work by Behrend *et al.* (1994) and discussed in greater detail in Chapter 6 of this thesis represents the first report that demonstrates a functional role for OPN in malignancy. Cells expressing antisense *opn* RNA had reduced tumor growth rates and reduced metastatic ability when tested in nude mice. Similar experiments by Gardner *et al.* (1994) and Su *et al.* (1995) using antisense *opn* RNA have confirmed the results of Behrend *et al.* (1994), suggesting that over-expression of OPN contributes functionally to the malignant properties of tumor cells.

A number of recent findings by Hwang *et al.* (1994a) and Feng *et al.* (1995) may shed some light on the mechanism by which OPN may help tumor cells be more malignant. Hwang *et al* (1994a) have demonstrated that OPN can inhibit production of nitric oxide (NO) in proximal tubule epithelial (PTE) cells from kidney, by inhibiting induction of inducible nitric oxide synthase (iNOS) mRNA. NO is a short-lived reactive metabolite that is a component of the oxidative burst of macrophages. Feng *et al.* (1995) have shown that *ras*-transformed mouse fibroblasts down-regulated for OPN by transfection with anti-OPN ribozyme expression vectors were more susceptible to killing by macrophages than cells expressing high levels of OPN. Cells down-regulated for OPN were also less tumorigenic and less metastatic than cells expressing high levels of OPN, when tested in nude mice. These findings have led Denhardt and Chambers (1994) to propose a mechanism for OPN function in malignancy: OPN secreted by tumor cells may serve to protect tumor cells against NO-mediated host cell cytotoxicity and thereby promote the survival of the tumor cells in the host. It is possible that OPN secreted by tumor cells may function as a signaling molecule in autocrine or paracrine signal transduction pathways and mediate its effects by binding to $\alpha_v\beta_3$ integrin receptors on target cells. $\alpha_v\beta_3$ and other integrin receptors have been shown to participate in signal transduction events (Seftor *et al.*, 1992; Felding-Habermann *et al.*, 1992; Clyman *et al.*, 1992; Miyauchi *et al.*, 1993; Yue *et al.*, 1994; Zimolo *et al.*, 1994; Liaw *et al.*, 1995;

Juliano, 1994; Clark and Brugge, 1995).

More recently a number of groups have examined OPN expression in human tumors. Brown and colleagues (1994) studied OPN expression and distribution in human carcinomas from a great variety of sources and found that there were substantial increases in *opn* mRNA in all 14 tumors examined and that there was intense labeling (by *in situ* hybridization) for *opn* mRNA in most (71 of 76) carcinomas. Surprisingly, in most carcinomas, macrophages associated with tumor cells, but not tumor cells themselves, were positive for *opn* mRNA. Carcinomas of the kidney and endometrium were the exception, where both tumor cells and macrophages stained positive for *opn* mRNA. Staining for *opn* mRNA was most intense at the tumor-stroma interface and in areas of tumor necrosis. Both tumor cells and macrophages stained positive for OPN protein, which led Brown *et al.* (1994) to propose that macrophage-derived OPN could bind to tumor cells and elicit a response that will affect the outcome of tumor progression. Two other studies have examined OPN expression in breast cancer. Hirota *et al.* (1995) observed OPN expression in cells that clustered around the necrotic foci within cancer cell nests, and identified these cells as macrophages. Bellahcene and Castronovo (1995) found undetectable levels of *opn* mRNA in normal mammary tissue, weak staining for *opn* mRNA in benign breast lesions, and strong expression of *opn* mRNA in *in situ* and invasive breast carcinoma lesions. No indication of the cell type that represents the source of OPN was made. Bellahcene and Castronovo (1995) concluded that OPN may play a role in the formation of microcalcifications in breast cancer, and that OPN may be involved in the preferred bone homing of breast metastases.

Saitoh *et al.* (1995) have examined the role of OPN in human gliomas. They report expression of *opn* mRNA in all glioma cell lines analyzed. Furthermore, expression of *opn* mRNA in human astrocytoma tissue correlated well with the degree of malignancy of the tumors.

An analysis of OPN levels in a series of lymph node negative breast cancers revealed OPN-immunopositivity in 20% of cancers analyzed (O'Malley *et al.*, 1995). OPN-immunopositivity was localized to the perinuclear region of the neoplastic cells with

focal macrophage positivity. A positive correlation was made between low levels of OPN-immunopositivity in tumor cells and survival of the patient.

A lot of data have been accumulated that suggest a role for OPN in the development of a malignancy. Work by Behrend *et al.* (1994) and discussed in detail in chapter 6 of this thesis has demonstrated that over-expression of OPN by tumor cells contributes functionally to the malignant properties of tumor cells. The discovery that OPN detected in human tumors is derived mainly from macrophages came as a surprise to many investigators. The role of macrophage-derived OPN in human tumors and its association with tumor cells is unclear. However, in those human breast tumors where OPN is also secreted by tumor cells, the expression of high levels of OPN correlates with poor survival prognosis of the patients (O'Malley *et al.*, 1995). Much remains to be learned about OPN, including its role in normal physiological processes and the detailed mechanisms of how it promotes malignancy.

1.6 ANTISENSE TECHNOLOGY

1.6.1 Introduction

Research using antisense nucleic acids as tools to study genes and the protein products they encode has developed rapidly over the past 10 to 15 years. Antisense nucleic acids are single-stranded RNA or DNA oligonucleotides that are complementary to specific sequences in a particular target gene. Antisense RNAs and DNAs provide the possibility of manipulating gene activity in a highly specific manner, the specificity resulting from complementary base-pairing between the target and antisense sequences (Hélène and Toulmé, 1990; Hélène, 1991). This approach to studying gene function by selectively inhibiting the production of a specific protein, has been successful in many instances, but can also be very challenging to accomplish, and the mechanism of action is not always as predicted (Denhardt, 1992). Nevertheless, considerable experimental data on the function of many genes have already been accumulated, and efforts are underway to develop this very promising technology into clinically applicable, therapeutic regimens that may some day aid in curing neoplastic, and infectious diseases (Rothenberg *et al.*, 1989; Stein and Cohen, 1989; Calabretta, 1991).

Several different approaches to inhibiting production of a protein by antisense agents have been developed and will be discussed here. They are: 1) antisense oligonucleotides, 2) antigene oligonucleotides, 3) antisense RNA, and 4) ribozymes.

1.6.2 Antisense Oligonucleotides

Antisense oligonucleotides are short sequences of single-stranded DNA (oligodeoxyribonucleotides) or RNA (oligoribonucleotides), usually 12 to 20 nucleotides in length, and are designed to be complementary to a specific target gene sequence. The oligonucleotides are synthesized *in vitro* and are usually added to the cell culture medium for cells grown *in vitro*, or administered by microinjection or liposome-mediated delivery (Hélène, 1991).

Antisense oligonucleotides are known to exert their inhibitory effect, in part, at the level of translation. They can target the translation start codon and inhibit binding of translation initiation factors, including the 40S ribosomal subunit (Hélène, 1991; Eguchi *et al.*, 1991). Alternatively, they can target coding sequences of the mRNA and induce RNase H to cleave the mRNA bound in the DNA:mRNA duplex (Hélène, 1991; Eguchi *et al.*, 1991).

Although the name "antisense" oligonucleotide implies that these agents are designed to target the mRNA of a particular gene and induce translational arrest, other possible mechanisms of action have been suggested, including: (1) interference with transcription by binding to single-stranded DNA, (2) inhibition of RNA splicing by binding to splicing sites in heteronuclear (hn) RNA, or (3) blocking export of processed RNA to the cytoplasm (Hélène, 1991; Zhang and Roth, 1994).

A major drawback in the use of oligonucleotides as inhibitors of gene expression is the inherently transient effect of these antisense agents after application onto the cells. Unmodified phosphodiester oligonucleotides are readily degraded by nucleases present in the serum of the culture medium, which can partly be overcome by heat inactivation and by modification of the oligonucleotides (e.g. phosphorothioates, methylphosphonates, α-anomers, base modifications, and 3′ or 5′ modifications) (Kopper and Kovalszky, 1994). Degradation of these modified oligonucleotides by nucleases within the cells does

occur, but at a significantly reduced rate relative to unmodified oligonucleotides. In addition, oligonucleotides are quickly diluted out during cell division. To overcome this problem, repeated or continuous applications of antisense oligonucleotides may be necessary. This, in turn, may lead to an unusually high pool of intracellular nucleotides, which could result in unexpected toxicity (Kopper and Kovalszky, 1994). The use of modified oligonucleotides brings about another potential complication which is the accumulation of modified bases within the cells that could be a potential source of mutagenesis when these modified bases are incorporated into the DNA (Kopper and Kovalszky, 1994).

Despite the current delivery, stability, and potential toxicity problems of unmodified and modified antisense oligonucleotides, they have been shown to effectively inhibit the activities of a number of oncogenes and proto-oncogenes such as *ras*, *src*, *raf*, *myc*, *myb*, *kit*, *fms*, *fes*, *fos*, and *abl* (reviewed by Hélène, 1991; Prochownik, 1992; Stein and Cheng, 1993). For the development of antineoplastic or antiviral drugs, the use of oligonucleotides (both antisense and antigene) appears to be the most promising choice for now, since the alteration of cells with genetically engineered expression vectors lends itself less readily to chemotherapeutic application.

1.6.3 Antigene Oligonucleotides

Antigene oligonucleotides differ from antisense oligonucleotides in the type of intracellular structure they are designed to target. Antisense oligonucleotides are designed to target the mRNA of a particular gene, while antigene oligonucleotides are designed to target the DNA sequences of a particular gene to inhibit gene expression directly at the level of transcription (Hélène *et al.*, 1992). Antigene oligonucleotides bind in a sequence-specific manner in the major groove of a double-stranded target DNA to form a triple helix (Hélène, 1991). This approach requires that the target gene sequence be accessible within the chromatin structure in the nucleus (Hélène, 1991). The antigene strategy makes use of the fact that recognition sites remain present in the major and minor grooves of the Watson-Crick base-paired, double-stranded DNA molecule (Hélène and Toulmé, 1990). Thymine can bond with adenine while the adenine is simul-

taneously involved in Watson-Crick hydrogen bonding with another thymine. Similarly, protonated cytosine can bind to the guanine of a Watson-Crick hydrogen bonded G-C base pair. Therefore, oligonucleotides containing thymine and cytosine should be able to recognize and bind to DNA stretches containing adenine and guanine, as demonstrated by Maher *et al.* (1989). The type of hydrogen bonds formed between a thymine or pronated cytosine in an oligonucleotide and the adenine or guanine of a DNA double helix are termed Hoogsteen base pairing and result in the formation of a local triple helix (Hélène, 1991). The stability of triple helices is sensitive to physiological pH, and can be enhanced by substituting 5-bromouracil and/or 5-methylcytosine for thymine and cytosine (Povsic and Dervan, 1989).

Antigene oligonucleotides can inhibit gene expression by one of two mechanisms: a) they can act as negative regulators of transcription by blocking access of sequence-specific DNA binding proteins such as enzymes or transcription factors to their specific recognition sequences, or b) they can function as transcriptional repressors by inhibiting RNA polymerase activity at the site of oligonucleotide binding and downstream thereof (Zhang and Roth, 1994).

Like other oligonucleotides, antigene oligonucleotides suffer from degradation by nucleases. The stability of antigene oligonucleotides can be improved by using nuclease-resistant α-oligonucleotides, in which the natural β-anomers of nucleotides have been substituted by the more stable α-anomers of nucleotide units, or by modifying the oligonucleotides with unnatural bases such as 5-methlycytosine (Gagnor *et al.*, 1987; Gee and Miller, 1992). Conjugation of oligonucleotides with intercalating agents, cross-linking agents, alkylating agents, or reactive groups such as cleaving agents can also improve the effectiveness of the oligonucleotides by permanently inactivating the DNA (Hélène, 1992).

One advantage antigene oligonucleotides have over the antisense approach, in theory, is the reduced number of target molecules that must be inactivated in order to inhibit expression of the gene (Murray and Crockett, 1992). Genes are present in low numbers in the cell, usually one or two copies, whereas mRNAs are present in abundant amounts. The major limitations of the antigene approach are: (1) difficulties in getting

antigene oligonucleotides into the nucleus, and (2) the lack of long stretches of purine nucleotides (adenine and guanine) in genes or their regulatory sequences, which are required for recognition by the oligonucleotides containing cytosine and thymine. A stretch of at least 17 purines is required to serve as target site (Hélène and Toulmé, 1989). A partial solution to this problem was presented by "alternate-strand multimeric crossover" oligonucleotides (Horne and Dervan, 1990). These oligonucleotides consist of two short (8- to 9-mer) pyrimidine oligonucleotides that are joined at their 3' ends and they are capable of simultaneously binding to a purine stretch on one DNA strand and a second purine stretch on the other strand. This strategy greatly increases the number of sequences that can be targeted by antigene oligonucleotides.

1.6.4 Antisense RNA

Antisense RNAs have been shown to occur naturally in both prokaryotic and eukaryotic cells, where they appear to play regulatory roles in a number of cellular processes such as DNA replication, transcription, RNA processing, and translation (Eguchi *et al.*, 1991; Krystal, 1992). Antisense RNAs can be generated artificially to aid in the study of cellular gene function. To this purpose the cDNA template of a chosen target gene or pieces thereof can be cloned into an expression vector in reverse orientation so that the complementary strand is transcribed into a non-coding antisense RNA that can hybridize with the target mRNA and interfere with its translation or stability (Izant and Weintraub, 1985). Expression of the antisense RNA can be either transient or stable depending on the type of transformation and the choice of vector. Different types of promoters can be selected that will dictate the levels of expression of the antisense RNA, the timing of expression, or the tissue specificity of antisense RNA expression. Furthermore, promoters that are inducible by a variety of agents, such as heavy metals, hormones, or elevated temperatures, are available to more closely control the parameters of antisense RNA expression (Weintraub, 1990).

Several different mechanisms of action of antisense RNA have been proposed (Zhang and Roth, 1994). These include: (1) inhibition of transcription, (2) blocking of RNA splicing or export to the cytoplasm, (3) inhibition of translation, and (4) cleavage

of the mRNA-antisense RNA duplex by RNase III.

Difficulties associated with antisense RNA include variations in expression levels and persistence, degradation by nucleases, and formation of self-inhibitory secondary and tertiary structures (Zhang and Roth, 1994). The expected RNA:RNA duplex formation depends on the linearly exposed portions of the mRNA and the antisense RNA, which may be complicated by secondary and tertiary structure formation.

An obvious advantage of antisense RNA expressed from transfected expression vectors over antisense oligonucleotides is the constitutive or inducible production of antisense RNAs within the cells, so that inefficient uptake and delivery do not present so significant a problem with this approach (Zhang and Roth, 1994).

As for antisense oligonucleotides, hybridization of antisense RNA with target mRNA and/or DNA can take place in the nucleus, indicating that inhibitory effects of the antisense RNA might occur prior to translation, possibly at the level of transcription, RNA processing, or export of the mRNA into the cytoplasm (Hélène and Toulmé, 1990).

Suppression of transcription or translation of oncogenes or proto-oncogenes by antisense RNA has been successfully demonstrated for c-*fos* (Ledwith *et al.*, 1990), c-*myc* (Sklar *et al.*, 1991), and K-*ras* (Mukhopadhyay *et al.*, 1991) with simultaneous reversion of the transformed phenotype of the target cells. In another example, down-modulation of tissue inhibitor of metalloproteinases (TIMP) was achieved by expression of antisense RNA from the mammalian expression vector pNMH in Swiss 3T3 cells, and resulted in cells with increased tumorigenic and metastatic potential (Khokha *et al.*, 1989).

1.6.5 Ribozymes

Ribozymes are naturally occurring RNA molecules with endogenous catalytic RNase activity (Cech and Bass, 1986; Cech, 1987). Ribozymes can mediate their own cleavage, as well as promote cleavage of other RNA substrates (Hélène and Toulmé, 1990). Among the different types of ribozymes are the tetrahymena group I introns, the hammerhead ribozymes, the hairpin ribozymes, and RNase-P (Murray and Crockett, 1992). The best characterized ones are the hammerhead ribozymes.

The hammerhead ribozyme consists of 3 base-paired stems, I, II, and III, and a catalytic core region in which 13 nucleotides are strictly conserved (Forster and Symons, 1987; Haseloff and Gerlach, 1988). The catalytic center catalyzes the cleavage of the target RNA immediately 3′ to any NUX sequence (where N is A, G, C, or U; and X is C, A, or U) in the target RNA sequence. In naturally occurring hammerhead ribozymes, such as those of a number of plant viroids and satellite RNAs, the catalytic reaction is intramolecular, since the target and catalytic strands are part of the same molecule (Symons, 1989). However, hammerhead ribozymes can be constructed so that the reaction proceeds in *trans* and any cloned RNA can be cleaved after the triplet NUX sequence (Haseloff and Gerlach, 1988). Specificity to the particular RNA can be achieved by inserting complementary recognition sequences into stems I and III of the hammerhead ribozyme. Following cleavage of the target RNA, the cleavage products can dissociate from the ribozyme, allowing the ribozyme to target and cleave another RNA molecule (Larson *et al.*, 1993). The efficiency with which this recycling occurs depends in part on the stability of the duplex formed between the ribozyme and the target RNA before and after cleavage of the target (Bratty *et al.*, 1993).

Since ribozymes cleave their substrate target RNA, they are expected to be more effective at inhibiting gene expression than conventional antisense RNA which bind to their target RNA reversibly. Some experiments (reviewed by Hélène and Toulmé, 1990) have shown that this is not necessarily the case. Like other RNA molecules, ribozymes in target cells suffer from rapid degradation by nucleases. Slow turnover and recycling of ribozymes as a result of strong binding between target RNA and ribozyme may also reduce their effectiveness. Another problem may be the formation of secondary structures due to folding of long RNA molecules, which may render the catalytic site inaccessible (Hélène and Toulmé, 1990).

CHAPTER 2

OBJECTIVES AND RATIONALE

The most difficult problem to overcome in cancer treatment is the accurate detection and effective treatment of metastases. Intensive research on the cellular and molecular events involved in tumor development and progression have provided us with a much better understanding of the processes involved in the generation of a malignancy.

The view that cancer is a regulatory disease in which the aberrant cells are no longer under the control of the cellular regulatory mechanisms, has gained wide acceptance (Nowell, 1986; Bishop, 1991). Activation of proto-oncogenes and/or inactivation of tumor suppressor genes can cause a break in the tightly controlled growth regulatory mechanisms of the cell. This in turn may upset the delicate balance between downstream effector genes, whose expression is regulated by proto-oncogenes and tumor suppressor genes (Aoyama and Klemenz, 1993; Bortner *et al.*, 1993; Chambers and Tuck, 1993). If the altered patterns of gene expression in the deregulated cells favor increased growth, tumor formation may result. Progressive evolution to an increasingly uncontrolled phenotype may give rise to invasive and metastatic properties and the development of metastatic disease (Nowell, 1983).

An experimental model system that exemplifies this theory is that of *ras* oncogene-induced malignancy. NIH 3T3 mouse fibroblast cells transformed with the human T24 bladder carcinoma H-*ras* oncogene exhibit the characteristics of a transformed cell line and are tumorigenic and metastatic in nude mice and chick embryos (Bondy *et al.*, 1985; Hill *et al.*, 1988; Chambers *et al.*, 1990a). Analysis of the gene expression patterns of normal and *ras*-transformed NIH 3T3 cells reveals an increase in expression of genes associated with increased growth and malignancy (i.e. cathepsin L, osteopontin) and a reduction in expression of genes associated with suppression of malignancy (i.e. tissue inhibitor of metalloproteinase, retinoblastoma) (Tuck *et al.*, 1991; Chambers *et al.*, 1992c). One of these genes, osteopontin (*opn*), is the focus of this study.

The overall goal of this study was to improve our understanding of OPN, a secreted phosphoprotein whose expression is tightly regulated, and that is present in a select set of normal tissues, many body fluids, and is also detected in some pathological

conditions, including cancer.

The observation that expression of *opn* was significantly increased in *ras*-transformed NIH 3T3 cells, raised the possibility that *opn* could be a *ras*-responsive gene and that expression of *opn* may be under direct control of *ras*. Since two conflicting reports declaring to have identified the mouse *opn* gene structure were reported in the literature, it was necessary to clarify this disparity and establish the correct *opn* gene structure before any studies on the regulation of this gene could proceed.

Objective 1 of this study was aimed at clarifying the structure of the mouse *opn* gene by testing both reported *opn* gene structures for their validity. Establishing the correct gene structure, including the locatic. of the first exon, the transcriptional start site, and the promoter, is essential before any conclusions from studies on the regulation of the gene can be deemed reliable.

Objective 2 of this study was to determine whether the levels of OPN protein and *opn* mRNA expressed by *ras*-transformed cells correlated with the levels of *ras* mRNA and the increase in metastatic ability of these cells.

Objective 3 of this study was to determine whether OPN contributes functionally to the malignant properties of tumor cells. Frequently, OPN has been shown to be over-expressed in transformed cells. However, it has never been established whether OPN secreted by tumor cells contributes functionally to the tumorigenic and metastatic properties of these tumor cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS, PLASMIDS, AND *IN VITRO* DNA MANIPULATIONS

The *Escherichia coli* strain used for maintenance of plasmids was HB101 (Dr. J. Koropatnick, London Regional Cancer Centre). Bacteria were grown in Luria-Bertani (LB) broth (Miller, 1972). Plasmids pGEM4-2arcDNA, pNMH, and pNMH-2ar antisense were a kind gift of Dr. A. M. Craig and Dr. D. T. Denhardt (University of Illinois, Il and Rutgers University, NJ, respectively). HB101 bacteria carrying plasmids were grown in LB media containing 35 μg/ml ampicillin, and were stored frozen at -70°C in LB media plus 35 μg/ml ampicillin plus 15% glycerol.

pGEM4-2arcDNA contains the complete coding region of *2ar* (murine osteopontin) plus a bit of 5' and 3' untranslated flanking sequence (nt -18 to + 1079 with respect to the translational start site) (Craig, 1989). pGEM4-2arcDNA was used for purification of the *2ar* insert.

pNMH is a mammalian expression vector containing the heavy metal-inducible promoter metallothionein-I (MT-I) (Khokha and Denhardt, 1987). The vector pNMH-2ar antisense (pNMH-asOPN) (Craig, 1989) contains the *2ar* cDNA in an antisense orientation cloned into a *Bam*HI site. Plasmid DNA was purified from log phase bacterial cultures using Qiagen spin columns and the recommended Qiagen protocol (Qiagen Inc., Chatsworth, CA).

Restriction endonucleases and other DNA modifying enzymes were purchased from Grand Island Biological Co. (GIBCO) (GIBCO Life Technologies, Burlington, ON) and Promega (Promega Corporation, Madison, WI) and were used according to the supplier's instructions.

3.2 MAMMALIAN CELL CULTURE

Cell lines, culture media, and sources used are listed in Table 3.1. For transfection studies, NIH 3T3 and PAP2 cells were used as the parental cell lines. NIH 3T3 cells were originally obtained from Dr. D. Fujita (University of Calgary Calgary, AB) (Jainchill *et al.*, 1969).

PAP0 is a polyclonal (pooled) population of H-*ras*-transformed NIH 3T3 cells that

Table 3.1

Mammalian Cell Lines

Cell Line	Description	Culture Medium	Reference
NIH 3T3	mouse fibroblast	DMEM + 10 % CS	Jainchill *et al.* (1969)
PAP2	T24 H-*ras*-transformed mouse fibroblast (pool)	DMEM + 10 % CS	Hill *et al.* (1988)
PAP0	T24 H-*ras*-transformed mouse fibroblast (pool)	DMEM + 10 % CS	Hill *et al.* (1988)
C2P0, C2P2, C5P0, C5P2	individual clones of T24 H-*ras*-transformed mouse fibroblasts	DMEM + 10 % CS	Hill *et al.* (1988)
PAP2 + pNMH-asOPN	individual clones of PAP2 transfected with antisense osteopontin	DMEM + 10 % CS + 200 μg G418 (active/ml)	generated in this study
J774A	mouse macrophage cell line	DMEM + 10 % FBS + 4.5 g/l glucose	Ralph *et al.* (1975)
IGR37	human melanoma cell line	alpha (-) MEM + 10 % FBS	Foa (1979)

was obtained after transformation of NIH 3T3 cells with the 6.6-kb H-*ras* T24 oncogene cDNA of plasmid pT24-C3 (Hill *et al.*, 1988). Plasmid pT24-C3 was generated by Pulciani *et al.* (1982) and was obtained from the American Type Culture Collection (ATCC, Rockville, MD). PAP2 is the same polyclonal cell population after two *in vivo* passages through the chick embryo (Hill *et al.*, 1988). C2P0 and C5P0 are individual clones of H-*ras*-transformed NIH 3T3 cells, and C2P2 and C5P2 are the same clones obtained after two *in vivo* passages through the chick embryo (Hill *et al.*, 1988). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO), supplemented with 10% calf serum (CS) (GIBCO).

PAP2-derived clones transfected with the expression vectors pNMH and pNMH-asOPN, were grown in DMEM plus 10% CS plus 200 μg Geneticin/G-418 sulfate (active/ml) (GIBCO).

The J774A mouse macrophage cell line was obtained from Dr. John Harris (London Regional Cancer Centre) (Ralph *et al.*, 1975). These cells were grown in DMEM plus 10% fetal bovine serum (FBS) (GIBCO) and 4.5 g/L of glucose.

IGR37 human melanoma cells were obtained from Dr. Liao (McMaster University, Hamilton, ON) (Foa, 1979). IGR37 cells were grown in minimum essential medium without ribonucleosides and without deoxyribonucleosides (alpha minus MEM) (GIBCO) supplemented with 10% FBS (GIBCO).

All cell lines were maintained at 37°C in an atmosphere of 5% CO_2 at subconfluent densities. Cells were passaged by trypsinization with 0.1% trypsin (Difco Laboratories, Detroit, MI) in citrate saline every 3-4 days, except for J774A, which are trypsin-sensitive and hence were scraped off the plastic with sterile cell scrapers (Falcon, Becton Dickinson, Lincoln Park, NJ). For long-term storage, cells were frozen in serum and 10% dimethyl sulfoxide (DMSO) and stored at -80°C or in liquid nitrogen.

3.3 TRANSFECTIONS

Transfections of PAP2 cells with pNMH or pNMH-asOPN were accomplished using the polybrene transfection procedure of Chaney *et al.* (1986). Four 100-mm plates (Falcon), each containing 4.5×10^5 cells/plate, were set up and incubated overnight at

37°C. The following day, 3 plates were treated with a mixture of 30 μg/plate of polybrene (Aldrich Chemical Co., Inc., Milwaukee, WI) and 0.5 μg/plate of DNA (pNMH or pMNH-asOPN) in DMEM plus 10% CS for 6-8 hours. One plate was treated with pNMH, while the other 2 were treated with pNMH-asOPN. The remaining control plate was left untreated. The cells on the 3 experimental plates were treated with 15% (v/v) DMSO in DMEM plus 10% CS for 4 min and incubated in DMEM plus 10% CS for 1.5 days. One and a half days later, on day 4, the medium on all 4 plates was changed to the selection medium, DMEM plus 10% CS plus 400 μg (active/ml) Geneticin (G-418). On day 9, fresh DMEM plus 10% CS plus 400 μg (active/ml) Geneticin (G-418) was added to all 4 plates. Once a week thereafter, the medium was changed to fresh DMEM plus 10% CS plus 200 μg (active/ml) Geneticin (G-418) to maintain selective pressure on arising colonies. As soon as colonies appeared, they were picked and frozen, as described in section 3.2, for later screening. A pooled population of all colonies from each plate was also obtained.

Transfection of NIH 3T3 cells with the 6.6-kb H-*ras* T24 oncogene were described in detail by Hill *et al.* (1988), but will be reviewed briefly since the series of 6 *ras*-transformed NIH 3T3 cells were used extensively in this study. Five x 10^5 NIH 3T3 cells were transfected with 1 μg H-*ras* T24 DNA plus 30 μg of carrier NIH 3T3 DNA by calcium phosphate precipitation (Hill *et al.*, 1988; Bondy *et al.*, 1985; Pulciani *et al.*, 1982). Control plates were treated identical except the H-*ras* T24 DNA was omitted. Individual, clonal foci of morphologically transformed cells (or pools of such foci) were isolated, briefly grown *in vitro* and analyzed by Southern blot analysis for the presence of the *ras* oncogene in the genomic DNA, by immunoblotting for expression of the *ras* p21 protein, and for their metastatic ability using the chick embryo assay (Hill *et al.*, 1988).

3.4 NUCLEIC ACID PURIFICATION

3.4.1 Purification of DNA and RNA from Cultured Mammalian Cells

DNA was isolated as described by Bondy and Denhardt (1983). Briefly, cells were harvested by trypsinization, washed twice with phosphate buffered saline (PBS) and

resuspended in DNA lysis buffer (0.1 M EDTA, 0.2 M Tris, pH 8.5). The mixture was passed through an 18-gauge needle several times to shear the DNA. SDS at a final concentration of 1% was added and the mixture was digested with 200 μg/ml of proteinase K overnight at 37°C. Membraneous material was precipitated with cold 5 M potassium acetate. Debris was removed by centrifugation (27000 x g for 15 min). Nucleic acids were precipitated by treatment with 2.5 volumes of 95% ethanol (EtOH) and 2 M ammonium acetate. RNA was digested with 100 μg/ml of RNase A (Boehringer Mannheim Canada, Laval, PQ) for 2 hours at 37°C. DNA was purified by one extraction with phenol/chloroform and two extractions with chloroform/isoamyl alcohol. DNA was precipitated as above, resuspended in TE (10 mM Tris.Cl [pH 8.0], 1mM EDTA [pH 8.0]) buffer, and stored at 4°C.

Cytoplasmic RNA was prepared as reported by Edwards and Denhardt (1985). Briefly, cells were harvested by trypsinization, washed once with cold PBS, and resuspended in TSM (30 mM Tris HCl [ph 7.6], 150 mM NaCl, 1.5 mM $MgCl_2$) buffer plus 0.2% Nonidet P40 (NP-40) (BDH Inc., Toronto, ON). Cells were disrupted in a glass homogenizer and nuclei were pelleted by centrifugation. The supernatant was mixed with an equal volume of lysis buffer (10 mM Tris HCl [pH 8.0], 7 M urea, 0.35 mM NaCl, 1 mM EDTA, 2% SDS), and RNA was extracted by two extractions with phenol/chloroform. RNA was precipitated twice with 3 M sodium acetate and 2.5 volumes of cold absolute ethanol overnight at -20°C. Total cytoplasmic RNA was resuspended in DEPC-treated (diethyl pyrocarbonate-treated) water and stored at -80°C.

When indicated, cells transfected with the expression vectors that carry the inducible metallothionein-I (MT-I) promoter (pNMH or pNMH-asOPN) were induced with 20 μM cadmium chloride ($CdCl_2$) for 4 hours prior to RNA extraction.

3.4.2 Purification of DNA and RNA from Primary Mouse Tumors

Primary tumors were generated by subcutaneous injection of cells transfected with pNMH and pNMH-asOPN into the right hind thigh of 4-5 weeks old, female nude mice as described later in section 3.14. Tumors were frozen in liquid nitrogen immediately after dissection. DNA from these tumors was prepared as follows. An approximately

1-cm^3 piece of tumor mass was removed from the frozen tumor sample, minced into small pieces with a razor blade, and homogenized in a glass homogenizer containing DNA lysis buffer. The mixture was passed through an 18-gauge needle several times to shear the DNA. SDS at a final concentration of 1% was added and the mixture was digested with 200 μg/ml of proteinase K at 37°C overnight. Extraction of DNA was performed as described in section 3.4.1.

RNA from primary tumors that were generated by subcutaneous injection of cells transfected with pNMH and pNMH-asOPN was prepared using the RNAzol B reagent (Biotecx Laboratories, Inc., Houston, TX). An approximate 1-cm^3 piece of tumor mass was removed from the frozen tumor sample and immediately placed into a chilled glass homogenizer containing RNAzol B. The tissue was homogenized, and RNA was extracted twice with chloroform following the manufacturer's protocol. The RNA was precipitated with isopropanol at -20°C for 1 h and with ethanol and sodium acetate overnight. RNA was resuspended in DEPC-treated water and stored at -80°C.

3.5 GEL ELECTROPHORESIS OF DNA AND RNA

DNA, in TE and loading buffer (30% glycerol, 0.25% bromophenol blue) was electrophoresed on 0.7% agarose (Seakem) gels (Mandel Scientific, Guelph, ON). For Southern blots shown in Figures 6.1 and 6.8, 10 μg/lane of DNA were electrophoresed, while for the Southern blot shown in Figure 4.3, 20 μg/lane of DNA were electrophoresed. The running buffer contained 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, and 0.5 μg/ml ethidium bromide. DNA was visualized and photographed under short wave ultraviolet light. Molecular weight markers used were the 0.12-21.2 kbp lambda DNA restriction fragments obtained from Boehringer Mannheim Canada. For isolation of DNA fragments from agarose gels, the whole band was excised from the gel with a scalpel and the DNA purified using a Prep-A-Gene kit from Bio-Rad Laboratories Ltd., Mississauga, ON.

Total cytoplasmic RNA (10 μg/lane), in DEPC-treated water plus 0.65X MOPS (BDH Inc.), 8.6% formaldehyde, 64.5% formamide plus loading buffer (1X MOPS, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol, and 50% glycerol) was

electrophoresed on a 1.1% agarose gel containing 6.8% formaldehyde under denaturing conditions (Maniatis, 1982). The running buffer contained 1X MOPS and 6.8% formaldehyde. Molecular weight standards were provided by a 0.24-9.5 kb RNA ladder obtained from GIBCO.

3.6 SOUTHERN AND NORTHERN BLOTTING ANALYSIS

Southern blots shown in Figures 6.1 and 6.8 were performed using a nitrocellulose membrane (0.45 μm, BA-85, Schleicher and Schuell, Keene, NH), since hybridization with only one probe was required. The Southern blot shown in Figure 4.3 was performed with GeneScreen Plus nylon membranes (New England Nuclear/DuPont Canada Inc., Mississauga, ON), since hybridization with more than one probe was required. Nylon membranes are stronger than nitrocellulose membranes and lend themselves better to repeated hybridizations.

In preparation for Southern blot transfer to a nitrocellulose membrane the DNA gel was first denatured in 1.5 M NaCl, 0.5 M NaOH two times for 15 min, and then neutralized in 0.5 M Tris-HCl, 3 M NaCl [pH 7.0] two times for 30 min. DNA was then transferred to nitrocellulose by the method of Southern (1975) in 20X SSPE (1X SSPE is 0.18 M NaCl, 0.01 M sodium phosphate [pH 7.4], 0.001 M EDTA [pH 7.4]. Filters were baked at 80°C for 2 h.

For the transfer of DNA to a GeneScreen Plus nylon membrane the gel was denatured in 0.5 M NaOH, 1.5 M NaCl two times for 20 min, and neutralized in 0.5 M Tris-HCl, pH 7.5, 3.0 M NaCl two times for 20 min. The transfer was carried out as described above for a nitrocellulose membrane, but using 10X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate) instead of 20X SSPE.

In preparation for the Northern transfer of RNA to a GeneScreen Plus nylon membrane, the formaldehyde gel was rinsed briefly in distilled water to remove excess formaldehyde. RNA was transferred to the nylon membrane in 10X SSC by capillary transfer. Filters were baked at 80°C for 2 h and RNA was crosslinked to the filter by exposure to ultraviolet light for 5 min.

3.7 DNA PROBES AND RADIOLABELING OF DNA PROBES

The *opn* (*2ar*) cDNA probe used was the 985 bp cassette of plasmid pGEM4-2arcDNA, (Craig, 1989). The *18S* rRNA cDNA probe was the 500 bp cassette of plasmid p100D9, obtained from Dr. D. T. Denhardt. These double-stranded DNA probes were radiolabeled by oligonucleotide-primed labelling with [α^{32}P]dCTP (NEN/Dupont) using a Pharmacia oligolabeling kit and procedure (Pharmacia P-L Biochemicals, Inc., Uppsala, Sweden). Probes were purified from unincorporated radionucleotide by spin column chromatography on a column of 1.5 ml of a Sephadex G-50 slurry (Pharmacia) (Maniatis et al., 1982) and counted in a liquid scintillation counter.

The *ras* probe was a 40-base, single-stranded, synthetic oligonucleotide antisense to a 5′ translated region of the human T24-H-*ras* gene (Pr-2, Oncogene Science, Inc., Manhasset, NY). The *β-actin* probe was a 40-base, single-stranded, synthetic oligonucleotide that recognizes exon 2 of the human *β-actin* and mouse *β-actin* gene (Oncogene Science, Inc.). The single-stranded *opn* probes (*opn* and *asopn*) were 40-base oligonucleotides complementary to the first 40 bases of exon 7 of the mouse *opn* gene (Figure 4.1; Behrend *et al.*, 1993; Craig and Denhardt, 1991). The *opn* probe specifically recognizes and hybridizes to *opn* mRNA, whereas the *asopn* probe specifically recognizes and binds to *opn* antisense RNA. These oligonucleotides were synthesized by the MRC Molecular Biology Core Facility, London Regional Cancer Centre. Probes A, B, and C are oligonucleotide probes also synthesized at the MRC Molecular Biology Core Facility, LRCC. Probe C is the 40-base *opn* probe mentioned above that specifically hybridizes to *opn* mRNA. It is complementary to the first 40 bases of exon 7 of the mouse *opn* gene, and has the sequence 5′ TCATGTGAGAGGT GAGGTCCTCATCTGTGGCATCAGGATA 3′. Probe A is a 35-base, single-stranded oligonucleotide that is complementary to the first 35 bases of exon 1 of the mouse *opn* gene as identified by Craig *et al.* (1989; 1991; Craig, 1989). Probe A has the sequence 5′ ACCCAAGCAAGGATGCTTCTTCAGTGTGAGCTGCT 3′. Probe B is a 30-base, single-stranded oligonucleotide that is complementary to a region in the center of exon 1 as assigned by Miyazaki *et al.* (1990). It has the sequence 5′ GTTGATGTCTTGTCA

GGTTCAAACACACTC 3'.

Single-stranded oligonucleotide probes were radiolabeled by 5' end-labeling with [γ^{32}P]ATP (NEN/Dupont) using a protocol from Oncogene Science. Probes were purified from unincorporated radionucleotide by spin column chromatography as above.

3.8 HYBRIDIZATION OF SOUTHERN AND NORTHERN BLOTS

Nitrocellulose filters were hybridized overnight under moderate stringency conditions (42°C, 50% formamide, 5X SSC, 5X Denhardt's solution (1X is 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin pentax fraction V), 20 mM sodium phosphate (monobasic), pH 6.5, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA (GIBCO), and denatured probe (1 x 10^6 cpm/ml)). Filters were washed 5 times with 2X SSC and 0.1% SDS at 25°C and 5 times with 0.1X SSC and 0.1% SDS at 45°C. Filters were then autoradiographed at -70°C, using Kodak XAR film (InterSciences Inc., Markham, ON).

Hybridization of GeneScreen Plus membranes was performed overnight under moderate stringency conditions (1 M NaCl, 50% formamide, 10% dextran sulfate, 1% SDS, 100 μg/ml denatured salmon sperm DNA, and denatured probe (1 x 10^6 cpm/ml)) at 42°C. For oligolabeled cDNA probes, membranes were washed 2 times in 2X SSC for 5 min at room temperature, 2 times in 2X SSC/1.0% SDS for 30 min at 65°C, and 2 times in 0.1X SSC/0.1% SDS for 30 min at room temperature. For single-stranded 5' end-labelled probes, the washing conditions were less stringent. Filters were washed 4 times briefly in 2X SSC at room temperature, 1X in 2X SSC/0.1% SDS for 30 min at 65°C, 1 times in 2X SSC/0.1% SDS for 5 min at room temperature, and 1 times briefly in 2X SSC at room temperature. Northern blots were autoradiographed as above.

3.9 METABOLIC RADIOLABELING OF SECRETED MAMMALIAN CELL PROTEINS

Secreted phosphoproteins were radiolabeled with [^{32}P]-orthophosphate (H_3PO_4) (NEN/DuPont) or Tran^{35}S-Label ([^{35}S]-L-methionine; [^{35}S]-L-cysteine) (ICN Biomedicals, Inc., Mississauga, ON), using a procedure modified from Craig *et al.* (1988). For the

experiments illustrated and discussed in chapter 5 of this thesis (Figures 5.3 and 5.4), 5 x 10^5 cells/well were plated. For the experiments illustrated and discussed in chapter 6 of this thesis (Figures 6.3A and 6.3B), 3.5 x 10^5 cells/well were plated. Cells were plated in 6-well multidishes (GIBCO) and incubated overnight at 37°C. The next day, cells were washed twice with phosphate- and serum-free MEM (GIBCO) or in methionine-free DMEM (GIBCO) plus 0.2% dialyzed FBS and incubated for 1 to 2 hours in the same medium. For experiments discussed in chapter 5 (Figures 5.3 and 5.4), the cells were then incubated for 4 hours in fresh phosphate- and serum-free MEM containing 10μCi/0.5 ml of carrier-free [^{32}P]-orthophosphate. For the experiments discussed in chapter 6 (Figures 6.3A and 6.3B), the cells were incubated for 4 hours in fresh phosphate- and serum-free MEM with 20 μM $CdCl_2$ plus 10 μCi/0.5 ml of carrier-free [^{32}P]-orthophosphate or in fresh methionine-free DMEM plus 0.2% dialysed FBS plus 20 μM $CdCl_2$ plus 50 μCi/0.5 ml of Tran^{35}S-Label. Triplicate samples of labeled media were collected and centrifuged at 10,000xg for 10 min to remove cell debris. ^{35}S-labeled OPN was precipitated with barium citrate (section 3.10). The supernatants were analyzed by polyacrylamide gel electrophoresis (section 3.11). Unlabeled control cells were incubated in fresh phosphate- and serum-free MEM plus 20 μM $CdCl_2$ or in methionine-free DMEM plus 0.2% dialysed FBS plus 20 μM $CdCl_2$, as above, and trypsinized; cell numbers were counted with a hemacytometer.

3.10 BARIUM CITRATE PRECIPITATION OF RADIOLABELED PROTEINS

Radiolabeled medium was precipitated prior to electrophoresis as described by Senger *et al.* (1988), by the addition of 1/10 volume sodium citrate solution (3.8 g [$Na_3C_6H_5O_7H_2O$]/100 ml H_2O) and 1/10 volume barium chloride solution (15 g [$BaCl_2H_2O$]/100 ml H_2O). Precipitated labelled proteins were eluted from the insoluble barium citrate pellet with electrophoresis sample buffer (1X is 62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.0025% bromophenol blue) containing 0.2 M sodium citrate. A 20-fold concentration of OPN was accomplished in lanes labeled *-P* (Figure 5.4) by precipitating OPN protein from culture medium which was 20 times the volume compared to the volumes loaded in lanes labeled *-T* and *-S*.

3.11 GEL ELECTROPHORESIS OF LABELED PROTEINS

Proteins were analyzed on denaturing SDS-polyacrylamide gels according to Laemmli (1970), using 4% stacking gels and 12% separating gels or occasionally 20%-5% gradient gels. Volumes of ^{32}P-or ^{35}S-labeled cell culture medium for loading on gels were standardized according to cell number. ^{35}S-labeled medium was precipitated with barium citrate prior to electrophoresis to concentrate the OPN protein. Molecular weight markers used were the high range ^{14}C-methylated protein standards (GIBCO). Gels containing ^{35}S-labeled samples were fluorographed with diphenyloxazole using the procedure of Bonner and Laskey (1974). Both ^{35}S- and ^{32}P-containing gels were dried for 90 min at 80°C in a gel dryer (Bio-Rad Laboratories Ltd.) and autoradiographed at -70°C, using Kodak XAR film (InterSciences Inc.).

3.12 EXPERIMENTAL METASTASIS ASSAY IN THE CHICK EMBRYO

One x 10^5 cells in 0.1 ml DMEM plus 10% CS were injected intravenously (i.v.) into the chorioallantoic membrane veins of 11-day-old chick embryos as described (Hill *et al.*, (1988); Chambers *et al.*, (1982)). Cells were not induced with cadmium chloride prior to *in vivo* analysis. At least 8 embryos/cell line were tested. Metastatic ability of the injected cells was determined 7 days after injection by means of the ouabain plating assay (Hill *et al.*, 1988; Chambers *et al.*, 1982). The assay makes use of the natural resistance of rodent cells to ouabain cytotoxicity relative to chick cells. Chick livers were dissociated into single cell suspensions which were plated in DMEM plus 10% CS plus 2 x 10^{-5} M ouabain (Sigma Chemical Co., St. Louis, MO), a concentration sufficient to kill chick cells but not rodent cells (Chambers *et al.*, 1982). Following a 10-14 day incubation period, colonies that grew *in vitro* were counted, and the number of viable rodent cells that had been present in the chick liver were calculated. Metastatic ability of cells in this assay has been shown to correlate well with experimental metastatic ability in mice (Chambers *et al.*, 1990).

3.13 EXPERIMENTAL METASTASIS ASSAY IN THE NUDE MOUSE

Nude mice (Harlan Sprague Dawley *nu/nu* females, 4-5 weeks old) were used to assay for experimental metastatic ability and tumorigenicity. Animals were cared for according to standards of the Canadian Council for Animal Care under a protocol approved by the University of Western Ontario Council on Animal Care. Experimental metastatic ability was assessed by i.v. injection into the tail vein of nude mice. One x 10^5 cells/mouse were injected in 0.2 ml Hanks' balanced salt solution (HBSS) (GIBCO). Cells were not induced with cadmium chloride prior to *in vivo* analysis. Groups of 5 mice were injected for each cell line. Four weeks after injection, the mice were sacrificed and dissected. Lungs, liver, heart, spleen and kidneys were fixed in 10% neutral buffered formalin. Lung metastases were counted under a dissecting microscope, and the other organs were examined for gross metastases by eye.

3.14 TUMORIGENICITY AND SPONTANEOUS METASTASIS ASSAY IN THE NUDE MOUSE

Tumorigenicity was assessed by subcutaneous (s.c.) injection of cells into the right hind thigh of groups of 5 mice. One x 10^5 cells/mouse were injected in 0.2 ml HBSS. Cells were not induced with cadmium chloride prior to *in vivo* analysis. Primary tumors were measured every second day (length and width) with calipers starting on day 7 post-injection. Mice were sacrificed when the tumor reached a cross-sectional area of ~5 cm^2 or when the mice began to succumb to the burden of the tumor. Primary tumors were dissected under sterile conditions and used for preparation of RNA and DNA (section 3.4.2). Lungs, liver, heart, spleen and kidneys were fixed in 10% neutral buffered formalin and examined for gross metastases by eye.

CHAPTER 4

THE STRUCTURE OF THE MOUSE OSTEOPONTIN GENE

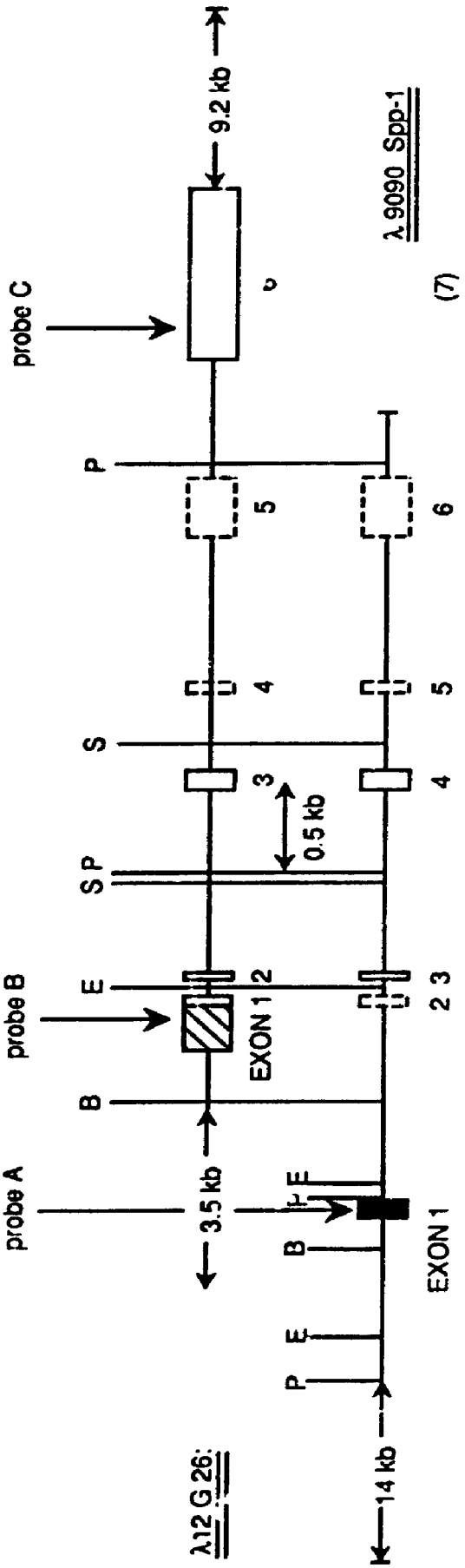
4.1. BACKGROUND

The gene structures for mouse, chicken, and human *opn* have been reported (Miyazaki *et al.*, 1990; Craig and Denhardt, 1991; Rafidi *et al.*, 1994; Hijiya *et al.*, 1994). The 5′ untranslated portion of the mouse *opn* gene including a substantial part of the promoter/enhancer region was independently characterized by Miyazaki *et al.* (1990) and Craig and Denhardt ((1991); Craig, 1989). The two groups came to different conclusions regarding the location of the promoter, the transcriptional start site, and the first exon (see Figure 4.1).

Miyazaki *et al.* (1990) isolated a phage EMBL3 genomic clone (12 G 26) from mouse liver containing a 4.8-kb region of the murine *opn* gene and 4.0-kb of 5′ flanking region and 9.4-kb of 3′ flanking sequences. Restriction mapping and nucleotide sequence analysis revealed that the mouse *opn* gene was comprised of six exons and five introns. "Exon 1" was described as being 250 bp long and containing an untranslated region of 196 bp. A single ATG initiation codon was reported to be present in "exon 1", which presumably encodes the full length of the signal peptide that destines the OPN protein for export to the outside of the cell. "Exon 5" was found to encode the 10 consecutive aspartic acid residues as well as the Gly-Arg-Gly-Asp-Ser (GRGDS) cell adhesion site, while "exon 6" was reported to encode the C-terminal half of osteopontin and to contain a 3′ non-coding region of 434 bp. Exons were determined by colinearity of the genomic sequences with the nearly full length sequence of the mouse *opn* cDNA clone MS21 (Miyazaki *et al.*, 1989).

A few differences in the nucleotide sequence of the 3′ untranslated region between the murine *opn* gene (Miyazaki *et al.*, 1990) and the mu o *n* cDNA (MS21) (Miyazaki *et al.*, 1989) were attributed by the authors to allelic polymorphism, since the genomic sequences originated from a BALB/c library, whereas the *opn* cDNA (MS21) was derived from ICR mice. Discrepancies between the murine *opn* cDNA (MS21) and the *opn* cDNAs reported by others (rat, Oldberg *et al.*, 1986; murine, Patarca *et al.*, 1989; murine, Craig *et al.*, 1989) were attributed to artifacts and sequencing errors. However, the short 5′ untranslated region (15 nucleotides) of the murine *opn* cDNA

Figure 4.1. Maps of the mouse genomic clones λ12 G 26 as reported by Miyazaki *et al.* (1990) and λ9090 Spp-1 as reported by Craig and Denhardt (1991). Clone λ12 G 26 was sequenced (Miyazaki *et al.*, 1990). Exons 1, 3, and 4 of λ9090 Spp-1 were sequenced, whereas exons 2, 5, and 6 were initially localized by Southern blot analysis (Craig, 1989), and were subsequently localized more accurately from the sequence data of Miyazaki *et al.* (1990). The two genomic clones were aligned by restriction enzyme sites (*B*, *Bam*HI; *E*, *Eco*RI; *P*, *Pst*I; *S*, *Sph*I) and sequence comparisons (Craig and Denhardt, 1991). The *cross-hatched* box constitutes the 5′ end of "exon 1" in the genomic clone λ12 G 26, and corresponds to the 5′ of an *opn* cDNA reported by Patarca *et al.* (1989). The *black box* represents the "first exon" as reported by Craig and Denhardt (1991). It corresponds to the 5′ ends of *opn* mouse and rat cDNAs reported by Craig *et al.* (1989) and Oldberg *et al.* (1986). The positions of the oligodeoxynucleotide probes A, B, and C used in this study are indicated by arrows (Behrend *et al.*, 1993).


λ12 G 26:
λ 9090 Spp-1
(7)
probe A
probe B
probe C
3.5 kb
9.2 kb
14 kb
0.5 kb
EXON 1
EXON 1
P
E
B
S
P
E
B
P
S
E
B
1
2
3
4
5
6
2 3
4
5

reported by Miyazaki *et al.* (1989) is present in its entirety in the longer 5′ untranslated region (69 nucleotides) of the murine *opn* cDNA described by Craig *et al.* (1989). The murine *opn* cDNA reported by Patarca *et al.* (1989) contained the longest 5′ untranslated region (222 nucleotides) and was used by Miyazaki *et al.* (1990) to deduce the 5′ of the *opn* mRNA.

Primer extension analysis and S1 nuclease mapping analysis were carried out to determine the transcriptional start site of the gene. Miyazaki *et al.* (1990) reported the identification of one major site of transcription initiation at +56 and several minor sites. The authors concluded that the transcription initiation sites among various cell types were different, and that monocytic cells, such as macrophages, mainly use the transcription initiation site at residue +56 (Miyazaki *et al.*, 1990).

Analysis of 0.75-kb of 5′ flanking region for the presence of *cis*-acting regulatory elements revealed a number of potential regulatory elements, including: several TATAA-like elements, several interferon regulatory factor-1 binding sequences, an inverted immunoglobulin octamer enhancer sequence, an erythroid-specific factor (GF-1) binding motif, an inverted GF-1 binding motif, and a glucocorticoid-response element (Miyazaki *et al.*, 1990). CCAAT consensus elements were not found, and evidence that any of the potential regulatory elements were functional was not provided.

Craig and Denhardt isolated a phage λ genomic clone (λ9090 Spp-1) containing 4.6 kb of the mouse *opn* gene including the first six exons, and an additional 15-kb of 5′ flanking DNA (Craig and Denhardt, 1991). The authors reported the existence of an additional 5′ "first exon" located upstream of "exon 1" reported by Miyazaki *et al.* (1990) (see Figure 4.1). According to data presented by Craig (1989) and Craig and Denhardt (1991), the mouse *opn* gene spans 5.7-kb and consists of 7 exons, the first exon being non-translated. Craig and Denhardt (1991) reported that there were no discrepancies in restriction enzyme sites or primary sequence between clones λ9090 Spp-1 and 12 G 26. The differences between the two reported *opn* gene structures rested: (a) in the assignment of exon 1, (b) the location of the promoter/enhancer region, and (c) the location of the transcriptional start site (Craig and Denhardt, 1991). The authors (Craig and Denhardt, 1991) attributed the discrepancy between the two reported *opn* gene

structures to the alignment of the genomic clones with independently isolated mouse *opn* (Spp-1) cDNA clones that differed in the 5′ untranslated sequence. The gene structure reported by Craig and Denhardt (1991) was based on the sequence of a cDNA isolated from mouse epidermal cells (Craig *et al.*, 1989). The 5′ end of this genomic clone (Craig and Denhardt, 1991) also corresponded to the 5′ end of a rat cDNA reported by Oldberg *et al.* (1986). In contrast, the gene structure reported by Miyazaki *et al.* (1990) was based on the sequence of a cDNA isolated from activated T-lymphocytes (Patarca *et al.*, 1989), which differed in the 5′ untranslated region from the cDNA clone isolated by Craig *et al.* (1989). Craig and Denhardt (1991) offered two possible explanations for the existence of two first exons: (1) different promoters might be used in epidermal cells and T cells, and (2) the T cell *opn* (Spp-1) cDNA (Patarca *et al.*, 1989) may be a truncated copy of a heterogeneous nuclear RNA still containing the first intron. Explanation 2 was reasonable since the cDNA isolated by Patarca *et al.* (1989) was derived from total cellular poly(A)$^+$ RNA, whereas the cDNA isolated by Craig *et al.* (1989) was derived from cytoplasmic poly(A)$^+$ RNA. Transient transfection assays, S1 mapping of the transcription start point, and sequence analysis demonstrated that the region upstream of "exon 1" as defined by Craig and Denhardt (1991) functions as a promoter in both epidermal and fibroblast cells, as well as in osteoblast-like cells (Noda *et al.*, 1990b).

4.2 OBJECTIVES

Two conflicting mouse *opn* gene structures, one derived from mouse macrophages (Miyazaki *et al.*, 1990), the other derived from mouse epidermal cells and also found to be correct for fibroblast cells (Craig and Denhardt, 1991; Craig *et al.*, 1989), had been reported. The two gene structures were identical in restriction enzyme sites and primary sequence, but differed in the assignment of the transcriptional start site, the promoter/enhancer region, and the location of the first exon. Work discussed in this chapter and published in *The Journal of Biological Chemistry* (Behrend *et al.*, 1993) was performed to resolve this discrepancy. Establishing the correct gene structure for mouse *opn* is essential if studies on the transcriptional regulation of the *opn* gene are to be

deemed reliable.

The objectives of this study were to determine: (1) which of the two reported gene structures represented the correct mouse *opn* gene structure, and (2) if the mouse *opn* gene structure and *opn* mRNA species expressed by fibroblasts differed from that of macrophages.

4.3 EXPERIMENTAL DESIGN

Northern blot analysis of the *opn* mRNA species expressed by fibroblast (NIH 3T3 and PAP2) and macrophage cell lines (J774A) should indicate whether the *opn* mRNA species predicted by the two *opn* gene structures actually exist. For this purpose, oligonucleotide probes to three regions of the reported mouse *opn* gene structures (see Figure 4.1) were generated and used to hybridize to the Northern blot of RNA from fibroblast and macrophage cell lines. The oligodeoxynucleotide probes used in this study are complementary to regions of the reported *opn* gene sequences and are described in detail in section 3.7.

Probe A is 35 nucleotides long and is complementary to the first 35 bases of "exon 1" as identified by Craig *et al.* (1989; 1991; (Craig, 1989)). Craig and Denhardt (1991) have shown that "exon 1" of Craig *et al.* (1989; 1991; (Craig, 1989)) lies immediately downstream of a functional promoter. The sequence recognized by probe A is *not* present in the *opn* mRNA proposed by Miyazaki *et al.* (1990), since it lies upstream of their putative promoter/enhancer region.

Probe B is 30 nucleotides long and is complementary to a region in the center of "exon 1" as assigned by Miyazaki *et al.* (1990). This sequence is present in the expressed *opn* mRNA sequence predicted by Miyazaki *et al.* (1990), but is *not* present in the *opn* mRNA reported by Craig *et al.* (1989).

Probe C is 40 nucleotides long and is complementary to the first 40 bases of "exon 6" as identified by Miyazaki *et al.* (1989; 1990). Probe C is also complementary to a 40-base sequence in the 3'region of the cDNA of Craig *et al.* (1989), and therefore served as positive control in this study.

Probe A can yield a signal with only the mRNA structure predicted by Craig *et*

al. (1989; 1991; Craig, 1989)), while probe B can yield a signal with only the mRNA structure predicted by Miyazaki *et al.* (1990). Probe C can yield a signal with both predicted *opn* mRNA structures.

Southern blot analysis of DNA from NIH 3T3, PAP2, J774A, and a human melanoma cell line, IGR37, was performed to determine the specificity of the probes A, B, and C, and find out whether these probes could recognize mouse, but not human, *opn* sequences.

4.4 RESULTS

4.4.1 Northern Blot Analysis

The results from the Northern blot analysis are illustrated in Figure 4.2. Hybridization of the Northern blot with probe A gave a strong band of 1.6-kb for PAP2 cells and a fainter band of 1.6-kb for NIH 3T3 cells. J774A cells also expressed *opn* mRNA as indicated by the band of 1.6-kb. Hybridization of the Northern blot with probe B showed no band for any of the three cell lines, even on very long exposure. Hybridization of the blot with probe C gave the same pattern as seen with probe A. Hybridization with *β-actin* confirmed equal loading of RNA for all three cell lines.

4.4.2 Southern Blot Analysis

The results from the Southern blot analysis are shown in Figure 4.3. Hybridization of three independent, identical Southern blots with probes A, B, and C yielded different banding patterns for each restriction enzyme and each probe. A single band was observed for all three murine cell lines (NIH 3T3, PAP2, and J774A) when cut with any one of the restriction enzymes and hybridized with probes A and B. The sizes of the bands detected with probes A and B were as predicted from the restriction map of the λ9090 Spp-1 *opn* clone reported by Craig *et al.* (1989; 1991). A major band plus a few nonspecific bands were observed on the blot hybridized with probe C. No band was observed for the human melanoma cell line IGR37, except that when IGR37 DNA was cut with *Pst*I and hybridized with probe C, a faint band slightly smaller than the band of the murine cell lines was visible. Hybridization with *β-actin* showed that

Figure 4.2. Northern blot analysis of *opn* and *β-actin* gene expression in mouse fibroblast and macrophage cell lines. Total cytoplasmic RNA (10 μg/lane) was separated, blotted and probed as described in the "Materials and Methods". Mouse fibroblast cell lines are NIH 3T3 and *ras*-transformed NIH 3T3 (PAP2). The mouse macrophage cell line is J774A. The same blot was sequentially stripped and reprobed with the oligonucleotide probes *A*, *B*, *C*, and *β-actin* as described under "Materials and Methods". Probes *A*, *B*, and *C* are as in Figure 4.1 and are described in section 3.7. Message sizes are: *opn*, 1.6-kb; and *β-actin*, 2.1-kb (Behrend *et al.*, 1993).

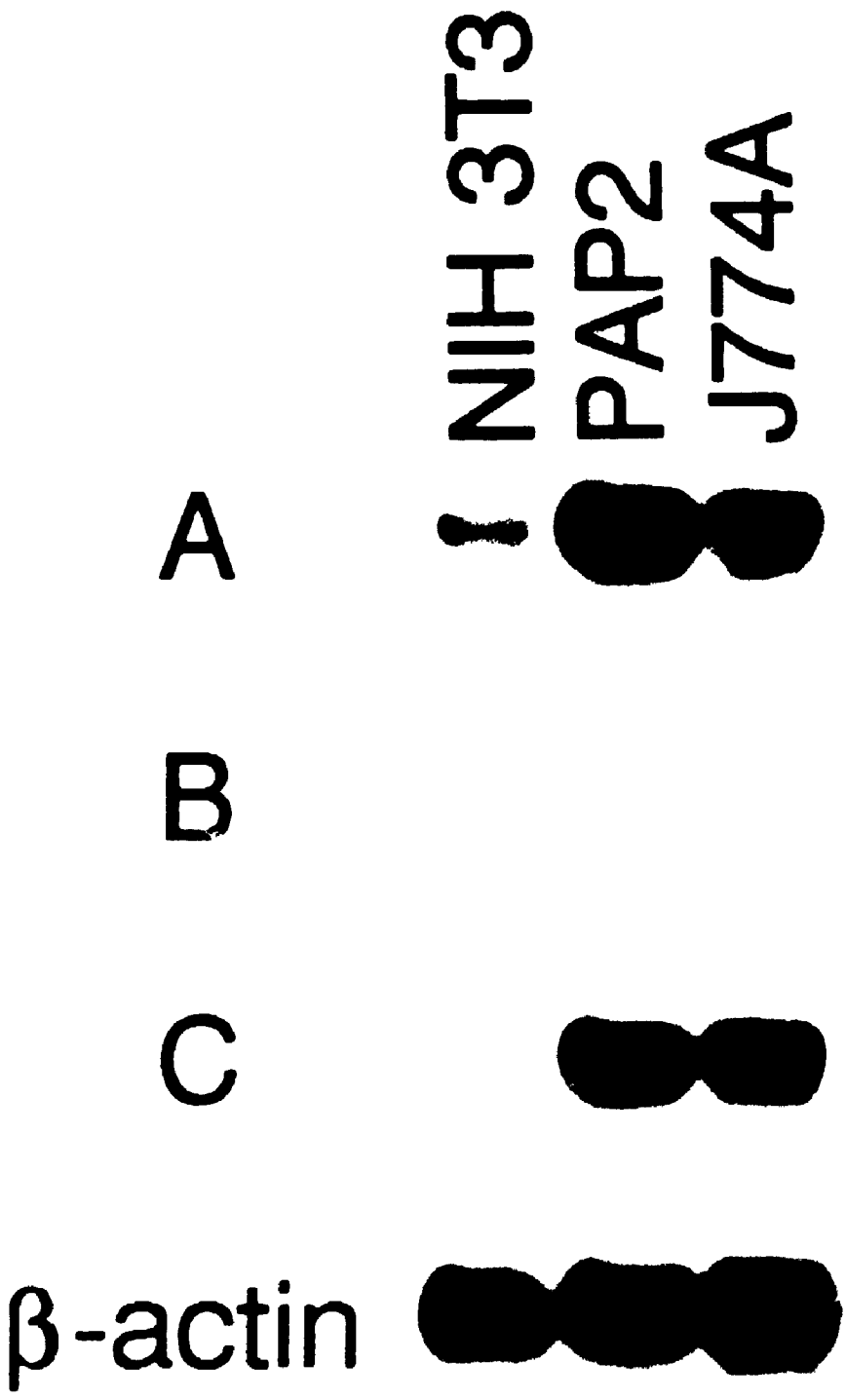
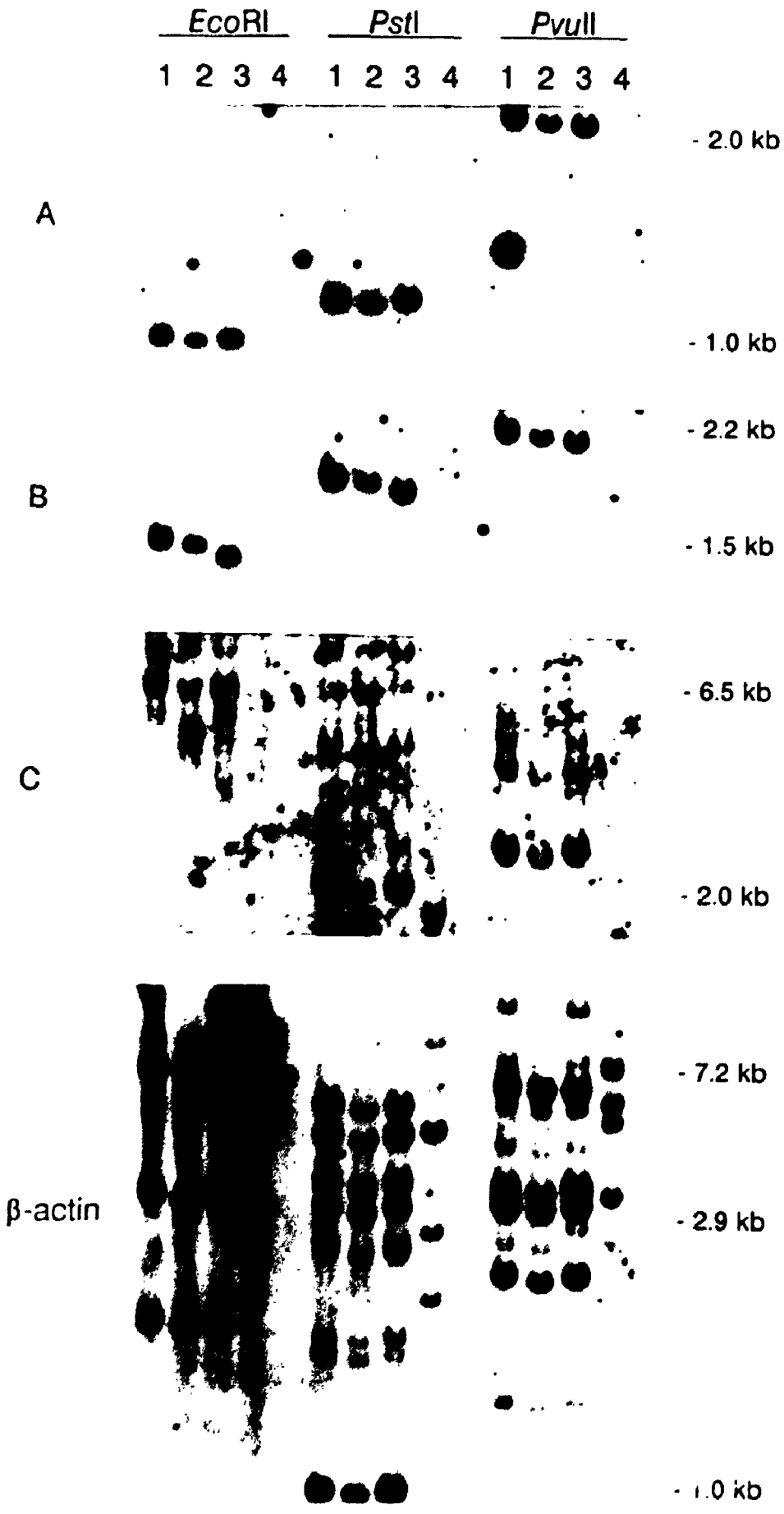

NIH 3T3
PAP2
J774A
A
B
C
β-actin

Figure 4.3. Southern blot analysis of the mouse *opn* gene in mouse fibroblast, mouse macrophage, and human melanoma cell lines. DNA (20 μg/lane) digested with *Eco*RI, *Pst*I, or *Pvu*II was separated, blotted and probed as described in the "Materials and Methods". NIH 3T3 and PAP2 (*ras*-transformed NIH 3T3 cells) are mouse fibroblast cell lines, J774A is a mouse macrophage cell line, and IGR37 is a human melanoma cell line. Three separate, identical Southern blots were probed with one of the oligonucleotide probes *A*, *B*, or *C*, and *β-actin* as described under "Materials and Methods". Probes *A*, *B*, and *C* are as in Figure 4.1 and are described in section 3.7. Lanes correspond as follows: *1*, NIH 3T3; *2*, PAP2; *3*, J774A; *4*, IGR37 (Behrend *et al.*, 1993).


EcoRI
PstI
PvuII
1 2 3 4 1 2 3 4 1 2 3 4
- 2.0 kb
A
- 1.0 kb
- 2.2 kb
B
- 1.5 kb
- 6.5 kb
C
- 2.0 kb
- 7.2 kb
β-actin
- 2.9 kb
- 1.0 kb

comparable amounts of DNA were loaded on all blots.

4.5 DISCUSSION

Two independently characterized gene sequences for the mouse *opn* gene had been reported (Craig, 1989; Miyazaki *et al.*, 1990; Craig and Denhardt, 1991). These sequences did not differ in their primary sequence or in restriction enzyme sites, but they did differ in the assignment of the promoter, the transcriptional start site, and exon 1 of the mouse *opn* gene. The analysis described here and published in *The Journal of Biological Chemistry* (Behrend *et al.*, 1993) established that the correct gene structure of mouse *opn* is that reported by Craig and Denhardt (1991).

Northern blot analysis (Figure 4.2) has shown that only probes A and C, but not B, can detect an abundant cytoplasmic RNA species of the size expected for *opn* mRNA in PAP2 fibroblast and J774A macrophage cells. The weaker signal observed for NIH 3T3 cells after hybridization with probes A and C lends support that the RNA species recognized by these probes is *opn*, since expression of *opn* is consistently lower in NIH 3T3 cells as compared to PAP2 cells (Craig *et al.*, 1988; Tuck *et al.*, 1991; Chambers *et al.*, 1992b; Behrend *et al.*, 1994). The results from the Northern blot analysis suggest that the sequences recognized by probes A and C, but not the sequences recognized by probe B, represent true coding sequences (exons) of the mouse *opn* gene. The results further illustrate that the *opn* mRNA species expressed by macrophages does not differ from the *opn* mRNA species expressed by fibroblasts in the three regions analyzed, since the expression patterns are identical for both cell types after hybridization with all three oligonucleotide probes.

Southern blot analysis was performed to determine: (1) whether probe B was functional and could indeed recognize mouse *opn* sequences, and (2) whether the sequences recognized by probe B represented genuine mouse sequences or unrelated non-murine sequences. Southern blot analysis (Figure 4.3) has shown that all three probes were capable of recognizing and hybridizing strongly to sequences in the mouse but not human genomic DNA, indicating that the sequences recognized by probes A, C, and particularly the sequences recognized by probe B, do indeed represent authentic mouse

sequences. These results also verified that probe B was functional and capable of hybridizing to mouse sequences, and further verified the authenticity of the absence of a signal upon hybridization of the Northern blot with probe B. The shift in the genomic fragment seen in the DNA of J774A cells (lane 3) upon hybridization with probe B relative to the genomic fragment for NIH 3T3 and PAP2 (lanes 1 and 2, respectively) may be attributed to the different genetic make-up of the mice that were used to establish these different cell lines. J774A cells were derived from a tumor which arose in a female BALB/c mouse in 1968, while NIH 3T3 cells were established from NIH Swiss mouse embryo cultures (American Type Culture Collection, 1992). However, only a complete nucleotide sequence analysis of the first two exons and the first intron of the *opn* gene in NIH 3T3 cells and J774A cells would clarify this discrepancy with certainty. The weak signal observed upon hybridization of probe C to human DNA was likely due to the strong sequence conservation in this region. There was 85% sequence identity between the mouse *opn* sequence recognized by probe C and the corresponding sequence in human *opn* cDNA compared to only 34% sequence identity for probe A (Kiefer *et al.*, 1989; Young *et al.*, 1990). The sequence complementary to probe B is not present in the reported human *opn* cDNA sequences (Kiefer *et al.*, 1989; Young *et al.*, 1990).

The results from both the Northern and Southern blot analyses demonstrate clearly that "exon 1" as reported by Craig (1989) and Craig and Denhardt (1991) is "exon 1" of the mouse *opn* gene. The results further show that "exon 1" as reported by Miyazaki *et al.* (1990) represents authentic mouse sequences, but a large portion of this sequence (the *cross-hatched* box, Figure 4.1) are non-coding intervening sequences, and the most 3′ sequences (*clear* box 3′ to the *cross-hatched* box, Figure 4.1) represent "exon 2" of the mouse *opn* gene. In addition, these results demonstrate that the mRNA species expressed by fibroblast cells and macrophage cells are not distinct, but similar.

The region upstream of "exon 1" as reported by Craig (1989) and Craig and Denhardt (1991) has been shown to function as a promoter in epidermal, fibroblast and osteoblast-like cells (Noda *et al.*, 1990b). Analyses of the *opn* gene structures of porcine (Zhang *et al.*, 1992), avian (Rafidi *et al.*, 1994), and human *opn* (Hijiya *et al.*, 1994) have revealed a 5′ "first exon" in other *opn* species, consistent with the results reported

here and with the 5′ "first exon" reported by Craig and Denhardt (1991) and inconsistent with the *opn* gene sequence proposed by Miyazaki *et al.* (1990).

A possible explanation for the erroneous conclusion of Miyazaki *et al.* (1990) involves the origin of the cDNA clone isolated by Patarca *et al.* (1989) and used by Miyazaki *et al.* (1990) to deduce the 5′ end of the *opn* mRNA. The cDNA clone isolated by Patarca *et al.* (1989) may actually have been an incompletely processed nuclear transcript that was present in the total RNA used in their original cloning. The short 5′ untranslated region of the cDNA reported by Miyazaki *et al.* (1989) is present in its entirety in the longer 5′ untranslated region described by Craig *et al.* (1989). The divergence between the *opn* cDNA sequences reported by Patarca *et al.* (1989) and Craig *et al.* (1989) occurs at the 5′ boundary of the second exon (Craig, 1989). It is possible that the *opn* species described by Miyazaki *et al.* (1990) may exist at low levels under some as yet poorly understood conditions. Zhang *et al.* (1992) did report that there was promoter activity exhibited by a region containing a TTTAAA sequence in the first intron that corresponded to the putative promoter reported for mouse *opn* in macrophages (Miyazaki *et al.*, 1990). However, primer extension and hybridization analysis of porcine and murine monocyte/macrophage and bone mRNA failed to reveal an *opn* mRNA transcript from this alternative promoter, suggesting that the same promoter regulates transcription of the *opn* gene in murine and porcine monocytes, macrophages, or bone cells.

This analysis of the mouse *opn* gene structure has demonstrated that the major *opn* mRNA species in murine fibroblasts and macrophages is that reported by Craig *et al.* (1989) and that the gene sequence reported by Craig (1989) and Craig and Denhardt (1991) is the correct mouse *opn* gene sequence. Establishment of the correct location of the promoter and the transcriptional start site of any gene is an absolute necessity before any future studies about the transcriptional regulation of the gene can be deemed reliable. One such study examining the transcriptional regulation of the mouse *opn* gene was published recently by Guo *et al.* (1995). In this study, the authors have identified a novel *ras*-activated enhancer (RAE) in the mouse osteopontin promoter and have described its interaction with a putative ETS-related transcription factor whose activity

correlates with the metastatic potential of the cell. This novel *ras*-activated enhancer is distinct from known *ras* response elements and appears to be responsible in part for the increased *opn* transcription observed in cells expressing activated *ras*. A ~16-kD protein was identified that complexed with DNA and this complex was generated at elevated levels by cell lines that are metastatic. Studies such as this one reported by Guo *et al.* (1995), which shed light on the transcriptional regulation of genes and which identify novel transcription factors, can not be deemed reliable if the structure of the gene, the location of the promoter and the transcriptional start site are not correctly identified. In this light, the work presented in this chapter of the thesis represents an important milestone in the elucidation of the structure and function of the *opn* gene and gene product.

4.6 CONCLUSIONS AND SIGNIFICANCE

The goal of this aspect of the study was to resolve the structure of the mouse *opn* gene. Two conflicting mouse *opn* gene structures had been reported. both claiming to have identified the mouse *opn* gene (Craig, 1989; Miyazaki *et al.*, 1990; Craig and Denhardt, 1991). The reported *opn* gene sequences did not differ in their primary sequence or in restriction enzyme sites, but they did differ in the assignment of the promoter, the location of the transcriptional start site, and the location of the "first exon". Another objective of this aspect of the study was to determine whether the *opn* gene structure and mRNA species were different in macrophages and fibroblasts, since one gene structure was derived from mouse macrophages (Miyazaki *et al.*, 1990), and the other from mouse epithelial cells, which was also found to be correct for mouse fibroblasts (Craig, 1989; Craig and Denhardt, 1991). Northern blot and Southern blot analyses with two probes that each hybridized to one of the two proposed first exons and a probe that hybridized to a region in the last exon of the mouse *opn* gene, which was not in dispute, have shown that "exon 1" as reported by Craig (1989) and Craig and Denhardt (1991) is "exon 1" of the mouse *opn* gene. In addition, these results have shown that the *opn* mRNA species expressed by fibroblasts and macrophages do not differ in the three regions analyzed in this study.

The work presented here has established unequivocally that the *opn* gene structure reported by Craig (1989) and Craig and Denhardt (1991) is the correct gene sequence for mouse *opn*. Furthermore, it represents an important milestone in the study of *opn*, since studies examining the transcriptional regulation of *opn* can only be deemed reliable if the correct gene structure of *opn* is established with certainty.

CHAPTER 5

EXPRESSION OF OSTEOPONTIN IN *RAS*-TRANSFORMED NIH 3T3 CELLS

5.1 BACKGROUND

Oncogenes have been used extensively to study the molecular mechanisms underlying transformation, tumor progression and metastasis (Cooper, 1990; Bishop, 1987; 1991; Spandidos and Anderson, 1989; Chambers *et al.*, 1992c; Chambers and Tuck, 1993). The model system used in this study is that of *ras* oncogene-induced tumorigenicity. Previously, Hill *et al.* (1988) reported that individual clones of NIH 3T3 cells transformed with the human bladder cancer (T24) H-*ras* oncogene were heterogeneous for both p21 protein (the *ras* oncoprotein) and experimental metastatic ability. However, p21 expression was a good indicator of metastatic ability of the clones, with metastatic ability increasing as p21 levels increased (Hill *et al.*, 1988). Selection of cells that were increased in their metastatic ability was accomplished by passaging individual clones once or twice through the chick embryo (Hill *et al.*, 1988). Selected clones were more metastatic in chick embryos and nude mice, and had increased levels of p21 expression, with the correlation coefficient being $r=0.85$ (Hill *et al.*, 1988; Chambers *et al.*, 1990a). Increased levels of p21 expression were the result of an increased proportion of p21-expressing cells (Chambers *et al.*, 1990a). These results suggested that quantitative increases in metastatic potential were the result of individual cells expressing increased amounts of p21 protein.

In the attempts to further analyze the molecular pathways linking increased p21 expression with increased metastatic ability, an hypothesis was put forward which proposed that *ras*-induced increases in metastatic ability might be the result of altered gene expression of *ras*-induced, metastasis-relevant genes (Chambers and Tuck, 1988). In support of this hypothesis, expression of a number of genes was reported to be increased in *ras*-transformed cells, including *opn*, cathepsin L, cathepsin B, and calcyclin, as well as type IV collagenase activity (Craig *et al.*, 1988; 1990; Chambers *et al.*, 1992c; Guo *et al.*, 1990; Tuck *et al.*, 1991). Furthermore, expression of other genes, including *jun*, *fos*, retinoblastoma (*rb*), tissue inhibitor of metalloproteinase (*timp*), as well as cystatin (cysteine proteinase inhibitors) activity were reported to be decreased

in *ras*-transformed cells (Tuck *et al.*, 1991; Chambers *et al.*, 1992c; Chambers and Tuck, 1993).

5.2 OBJECTIVE

Oncogenes are believed to promote tumor development and metastasis by perturbing cellular signal transduction pathways, resulting in: (a) activation of genes that promote malignancy and (b) inactivation of genes that inhibit malignant behavior. One gene whose expression was increased in *ras*-transformed cells was identified as *opn* (Craig *et al.*, 1988; 1990). OPN is a secreted $\alpha_v\beta_3$ integrin-binding cell adhesion protein, that has been linked to transformation on a number of occasions (see chapter 1 section 1.5.12 for review). The objective of this study was to determine whether *opn* mRNA expression correlated with expression of *ras* mRNA and with the metastatic potential of *ras*-transformed NIH 3T3 cells.

5.3 EXPERIMENTAL DESIGN

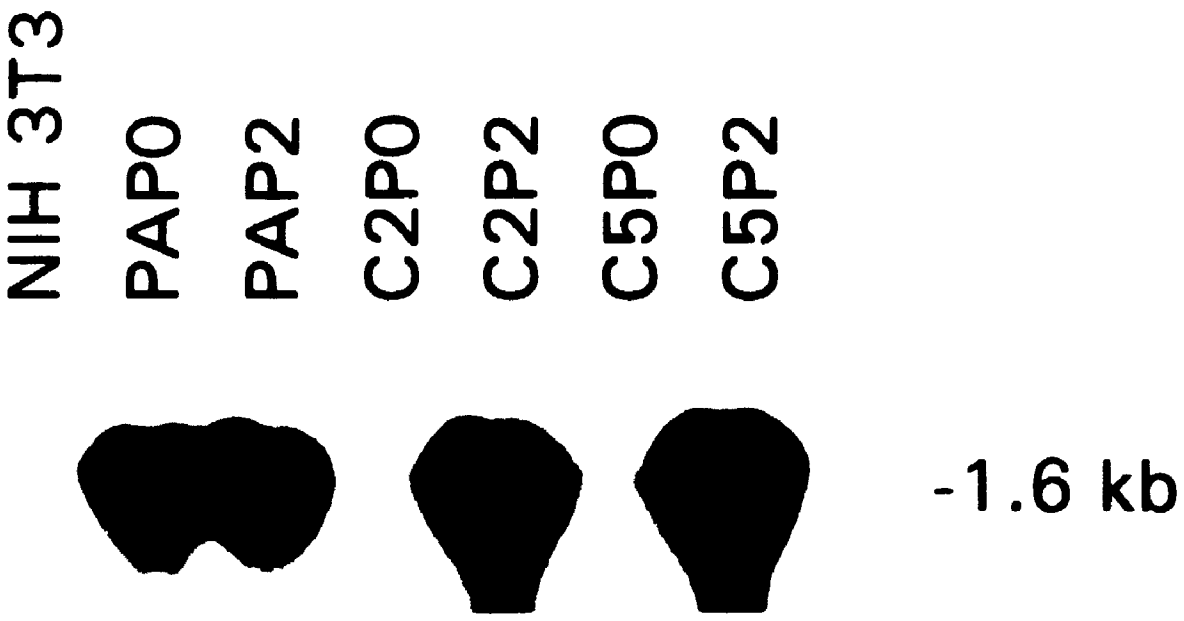
NIH 3T3 cells plus a series of 6 *ras*-transformed NIH 3T3 cell lines, generated by Hill *et al.* (1988), were analyzed for their levels of *opn* mRNA expression and OPN protein secretion. The expression levels of *ras* mRNA and the metastatic potential of these 7 cell lines have been reported previously (Chambers *et al.*, 1992c; Hill *et al.*, 1988). Expression levels of *ras* mRNA (Chambers *et al.*, 1992c) and *opn* mRNA were compared to determine if a positive correlation existed between *ras* and *opn*. Metabolically radiolabeled secreted OPN protein was analyzed by SDS-PAGE analysis to determine if levels of secreted OPN protein paralleled the levels of *opn* mRNA expression, *ras* mRNA expression, and the malignant properties of the tumor cells.

5.4 RESULTS

5.4.1 Expression of *opn* mRNA in *ras*-Transformed NIH 3T3 Cells

As illustrated in Figure 5.1, expression of the 1.6-kb murine *opn* mRNA was high in PAP0, PAP2, C2P2, and C5P2 cells. These cells are *ras*-transformed NIH 3T3 cells that express high levels of p21 protein and that are highly metastatic in both chick

Figure 5.1. Northern blot analysis of *opn* mRNA levels in NIH 3T3 cells and six *ras*-transformed NIH 3T3 cell lines. Total cytoplasmic RNA (10 μg/lane) was separated, blotted and probed with an oligolabeled *opn* (*2ar*) cDNA probe as described in the "Materials and Methods". NIH 3T3, C2P0, and C5P0 cells are non- or poorly-metastatic. PAP0, PAP2, C2P2, and C5P2 cells are metastatic. *Opn* message size is 1.6-kb. The experiment presented here was performed by Sylvia M. Wilson (Chambers, Behrend, Wilson, and Denhardt, 1992b).


NIH 3T3
PAP0
PAP2
C2P0
C2P2
C5P0
C5P2
-1.6 kb

embryos and nude mice (Hill *et al.*, 1988; Chambers *et al.*, 1990a). In contrast, NIH 3T3 cells, the non-metastatic control cell line, which does not express detectable levels of p21 protein, also showed undetectable levels of *opn* mRNA. Furthermore, C2P0 and C5P0, which are *ras*-transformed NIH 3T3 cells that express low levels of p21 protein and which are poorly metastatic in chick embryos and nude mice, expressed undetectable levels of *opn* mRNA. In an attempt to detect expression of *opn* mRNA in the poorly metastatic cells, the Northern blot (Figure 5.1) was over-exposed, giving rise to over-exposed bands for the metastatic cells.

A comparison of the levels of expression of *opn* mRNA (Figure 5.1) with those of *ras* mRNA (Chambers *et al.*, 1992c) in these cells resulted in a plot as shown in Figure 5.2. Expression levels of both *opn* and *ras* mRNA were normalized to β-actin. A strong correlation was observed between expression of *ras* mRNA and *opn* mRNA (correlation coefficient r=0.85). Cells which were poorly metastatic (NIH 3T3, C2P0, C5P0) expressed low levels of both *ras* mRNA and *opn* mRNA, while cells shown to be highly metastatic (PAP2, C2P2, C5P2) expressed high levels of *ras* mRNA and *opn* mRNA. The exception was PAP0 which was metastatic and expressed high levels of *opn* mRNA, but had relatively low levels of *ras* mRNA compared to PAP2 cells.

5.4.2 Secretion of OPN Phosphoprotein by *ras*-Transformed NIH 3T3 Cells

An analysis of the OPN protein levels secreted by these cells showed a pattern similar to that of the *opn* mRNA expression levels. A single band of apparent molecular weight of 51-kD was observed for each cell line (Figure 5.3). The amount of secreted OPN protein was low in NIH 3T3 cells and the poorly metastatic cell lines C2P0 and C5P0, and high in the highly metastatic cell lines PAP0, PAP2, C2P2, and C5P2. It was shown previously by immunoprecipitation that the single major band of secreted phosphoprotein in *ras*-transformed NIH 3T3 cells is OPN (Craig *et al.*, 1988). The apparent discrepancy between the levels of *opn* mRNA levels expressed by NIH 3T3 cells (Figure 5.1) and the levels of secreted OPN protein produced by NIH 3T3 cells (Figure 5.3) in these experiments may be due to timing of the experiments and possibly growth conditions. Northern blot analysis of *opn* mRNA expression and SDS-PAGE

Figure 5.2. Relationship of *ras* mRNA expression and *opn* mRNA expression in NIH 3T3 cells and six *ras*-transformed NIH 3T3 cell lines. Cell lines are as in Figure 5.1. *Open circles* denote the cell lines that are non- or poorly-metastatic; *closed circles* denote the cell lines that are metastatic. Data are from densitometric scans of Northern blots probed for *ras* and *opn* expression and normalized to β-actin. The dotted line is the linear regression of these data; correlation coefficient (r) is 0.85. (Chambers, Behrend, Wilson, and Denhardt, 1992b).

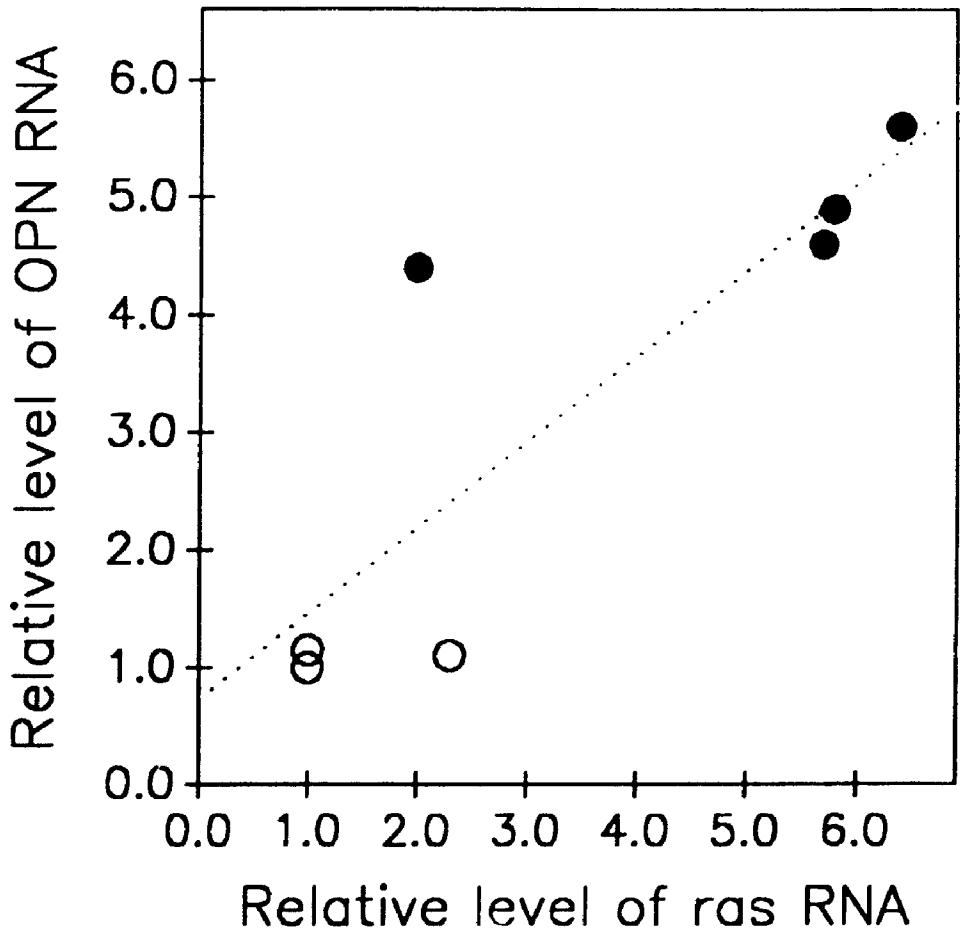
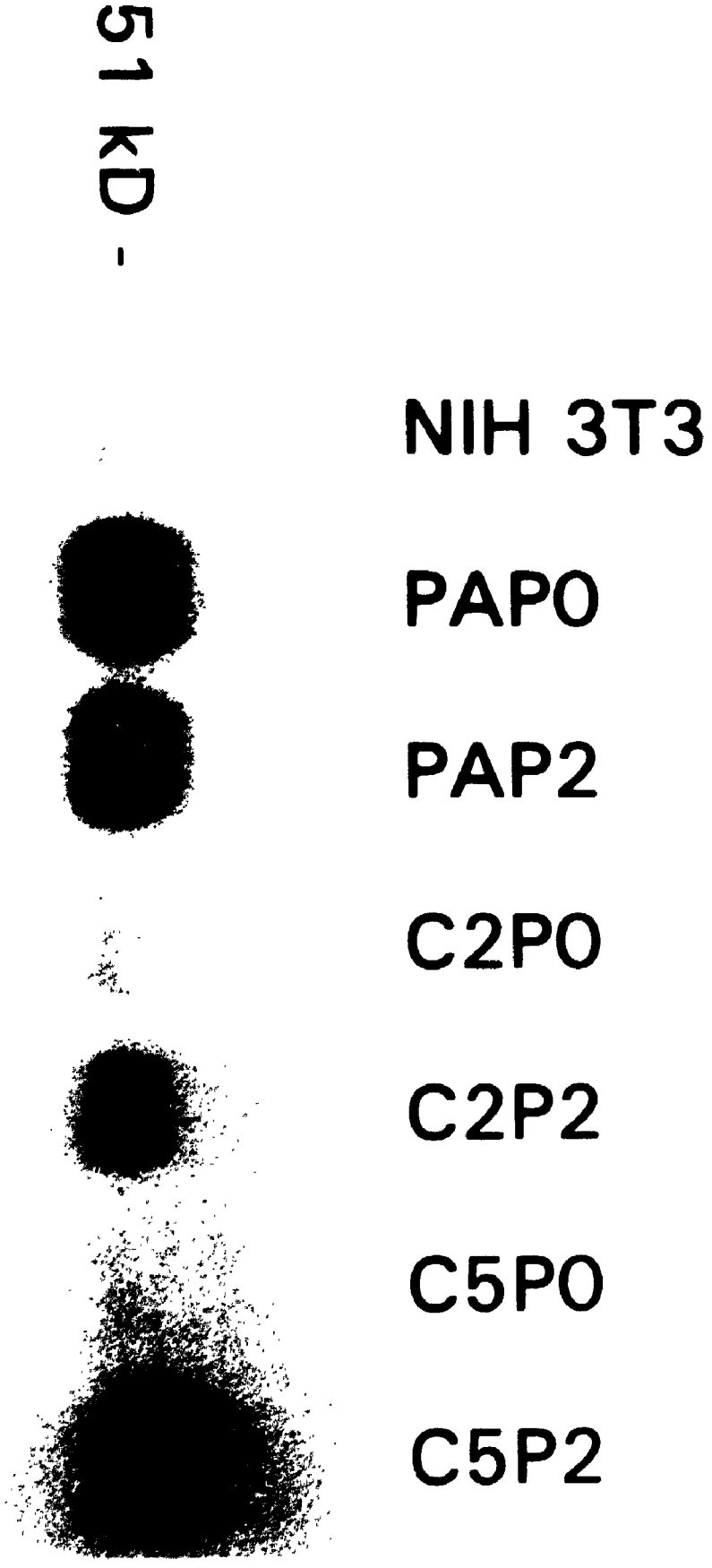

Relative level of OPN RNA
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0
Relative level of ras RNA

Figure 5.3. SDS-polyacrylamide gel electrophoresis of ^{32}P-labeled, secreted phosphoproteins from NIH 3T3 cells and six *ras*-transformed NIH 3T3 cell lines. Cells were metabolically labeled with [^{32}P]-orthophosphate, and medium was electrophoresed on a 12% SDS-polyacrylamide gel with volumes loaded per lane standardized to equal number of labeled cells, as described in the "Materials and Methods". NIH 3T3, C2P0 and C5P0 cells are non- or poorly-metastatic. PAP0, PAP2, C2P2, and C5P2 cells are metastatic. A single ^{32}P-labeled band was observed, at an apparent molecular weight of 51-kD (Chambers, Behrend, Wilson, and Denhardt, 1992b).


51 kD -
NIH 3T3
PAP0
PAP2
C2P0
C2P2
C5P0
C5P2

analysis of OPN protein secretion were done at different time points and with different batches of cells. In addition, expression of *opn* can easily be induced by addition of serum to the growth medium prior to RNA extraction or protein analysis.

5.4.3 Barium Citrate Precipitation of Secreted, Metabolically Radiolabeled OPN Protein

Barium citrate has been used previously by Senger *et al.* (1988, 1989b) to precipitate OPN protein from human serum and milk, and was used here to visualize the difference in OPN protein secretion from normal and *ras*-transformed cells (Figure 5.4). Presumably, the barium cation can mimic calcium and bind to OPN via the potential calcium binding site, or via the stretch of aspartic acid residues (Senger *et al.*, 1988). Cell culture medium from NIH 3T3 and PAP2 cells was analyzed. The lanes designated *-T* represent total unprecipitated medium, while *-P* represents cell culture medium that was precipitated with barium citrate and concentrated about 20-fold compared to the volume loaded in lanes labeled *-T*. Lanes labeled *-S* are the supernatant remaining after barium citrate precipitation.

A single band of 58-kD was detected for NIH 3T3 cells only when the cell culture medium was precipitated with barium citrate (NIH 3T3-P), but not in total unprecipitated medium (NIH 3T3-T), and not in the supernatant left after the precipitation (NIH 3T3-S). In contrast, a single band of 58-kD was observed for PAP2 cells in both the total unprecipitated medium (PAP2-T) and after precipitation with barium citrate (PAP2-P). No band was observed in the supernatant left after precipitation (PAP2-S). Most notably, the band representative of OPN protein secreted by PAP2 cells into the culture medium (PAP2-T) was more intense than the band for OPN protein secreted by NIH 3T3 cells and precipitated with barium citrate (NIH 3T3-P). The band representative of OPN protein secreted by PAP2 cells and precipitated with barium citrate (PAP2-P) was the most intense band of all.

The difference in apparent molecular weight of the OPN protein in Figure 5.3 (51-kD) and Figure 5.4 (58-kD) can be attributed to differences in acrylamide concentration and cross-linking in the two separate gels. Figure 5.3 is a 12% gel, while

Figure 5.4. Barium citrate precipitation and SDS-polyacrylamide gel electrophoresis of ^{32}P-labeled, secreted phosphoproteins from NIH 3T3 cells (non-metastatic) and PAP2 cells (metastatic). Cells were metabolically labeled with [^{32}P]-orthophosphate, and medium was electrophoresed on a 20%-5% gradient SDS-polyacrylamide gel as described in the "Materials and Methods". Lanes labeled -*T*; total medium, with volume loaded per lane standardized to equal numbers of labeled cells. Lanes labeled -*P*; 20X volume (as in -*T* lanes) was subjected to barium citrate precipitation and run on the gel. Lanes labeled -*S*: supernatant remaining after barium citrate precipitation; volume equal to lanes labeled -*T*. Apparent molecular weight of OPN in this gradient gel system was 58-kD (Chambers, Behrend, Wilson, and Denhardt, 1992b).

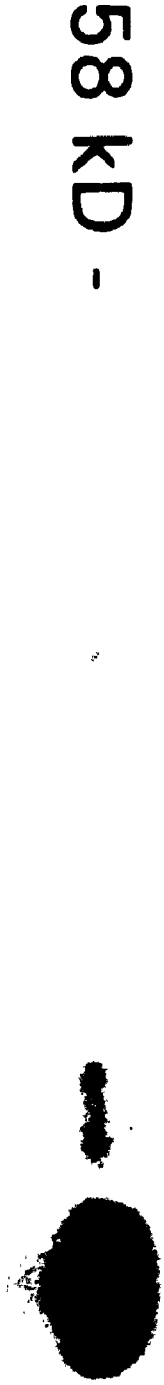

58 kD -
NIH 3T3-T
NIH 3T3-P
NIH3T3-S
PAP2-T
PAP2-P
PAP2-S

Figure 5.4 is a 20-5% gradient gel. This variation in apparent molecular weight has been reported previously, and is believed to represent the anomalous migration of this highly negatively charged protein (Butler, 1989; Denhardt and Guo, 1993). Other factors that can affect the apparent molecular weight of OPN include post-translational modifications such as the degree of phosphorylation (Nagata *et al.*, 1989; Kasugai *et al.*, 1991).

5.5 DISCUSSION

Previously, a number of studies had shown that *ras* oncogenes can promote the metastatic ability of transformed cells (Bondy *et al.*, 1985; Thorgeirsson *et al.*, 1985; Muschel *et al.*, 1985; Greig *et al.*, 1985; Bradley *et al.*, 1986; Pozzatti *et al.*, 1986). The metastatic ability of a series of individual clones of *ras*-transformed NIH 3T3 cells was heterogeneous, but correlated with the levels of p21 expression (Hill *et al.*, 1988). It was proposed that the *ras*-induced increases in metastatic ability might be due to altered gene expression of *ras*-induced genes that can promote or inhibit metastasis (Chambers and Tuck, 1988). One of these *ras*-induced genes was identified as *opn* (Craig *et al.*, 1988; 1990).

The goal of this study was to determine whether expression of *opn* mRNA correlated with expression of *ras* mRNA, and with the metastatic potential reported for these *ras*-transformed cells.

Analysis of *opn* mRNA expression (Figure 5.1) and secreted OPN protein (Figure 5.3) in a series of *ras*-transformed NIH 3T3 cells that have been analyzed previously for their *ras* mRNA expression (Chambers *et al.*, 1992c) and their metastatic potential (Hill *et al.*, 1988) has shown that *opn* mRNA expression correlated with the levels of p21 protein and with the metastatic potential of the tumor cells, and that the levels of secreted OPN protein paralleled the *opn* mRNA expression pattern. Highly metastatic cells expressed high levels of p21 protein (Hill *et al.*, 1988), high levels of *opn* mRNA (Figure 5.1), and high levels of OPN protein (Figure 5.3). Poorly metastatic cells expressed low levels of p21 protein (Hill *et al.*, 1988), low levels of *opn* mRNA (Figure 5.1), and low levels of OPN protein (Figure 5.3). PAP0 cells were the exception, being

highly metastatic in spite of low levels of *ras* expression (Hill *et al.*, 1988). This cell line is a pooled population of many different clones with variable levels of *ras* expression and variable metastatic abilities. Apparently the average levels of *ras* expression of this pooled population were low, while the high metastatic potential of some clones outweighed the low metastatic ability of other clones.

A comparison of the levels of secreted OPN protein produced by these cells (Figure 5.3) with the metastatic ability of these cells (Hill *et al.*, 1988) has revealed the following ranking orders:

(1) secreted OPN protein: PAP0 > PAP2 > C5P2 > C2P2 > NIH 3T3 > C2P0 > C5P0, and (2) metastatic ability: PAP2 > PAP0 > C5P2 > C2P2 > C5P0 > C2P0 > NIH 3T3. These rankings are in agreement with the above observations that metastatic cells (PAP0, PAP2, C5P2, and C2P2) express increased levels of *ras* and secrete elevated levels of OPN protein when compared to poorly metastatic cells (NIH 3T3, C2P0, and C5P0) which express lower levels of *ras* and secrete lower levels of OPN protein.

A comparison of the expression levels of *ras* and *opn* mRNA resulted in a similar ranking order for both genes: PAP2 > C2P2 > C5P2 > PAP0 > C2P0 > NIH 3T3 > C5P0. The strong correlation (r=0.85) (Figure 5.2) between levels of *ras* mRNA expression and *opn* mRNA expression indicated a direct relationship between *ras* and *opn*, and suggested that *opn* may indeed be a *ras*-responsive gene. In support of this hypothesis is the recent identification of a *ras*-activated enhancer (RAE) in the mouse *opn* promoter (Guo *et al.*, 1995).

Barium citrate precipitation of secreted OPN protein from the culture medium (Figure 5.4) was used to determine the difference in OPN protein secretion from normal and *ras*-transformed NIH 3T3 cells, and to illustrate that NIH 3T3 cells do indeed secrete OPN protein, although in very low amounts. OPN protein was likely too dilute in the labeled culture medium of NIH 3T3 cells (NIH 3T3-T) to be detected by SDS-PAGE analysis. Concentration of the OPN protein from the culture medium by a factor of ~20-fold, was sufficient to visualize the protein on the gel (NIH 3T3-P), and was able to demonstrate that NIH 3T3 cells do indeed secrete OPN protein into the culture medium. OPN protein was detectable in labeled culture medium from PAP2 cells (PAP2

-T), and concentration of OPN protein by ~20-fold (PAP2-P) gave an indication of the difference in OPN secretion between non-tumorigenic NIH 3T3 cells (NIH 3T3-P) and metastatic PAP2 cells (PAP2-P). The absence of a band for OPN protein in the supernatant left after barium citrate precipitation of PAP2 culture medium (PAP2-S) indicates the efficiency of this procedure in precipitating OPN protein.

Taken together, the results presented in this section of the thesis illustrate the difference in OPN production by non-transformed (NIH 3T3) and *ras*-transformed (PAP2) cells. In addition, the results demonstrate a positive correlation between expression of *opn*, *ras*, and the metastatic potential of *ras*-transformed NIH 3T3 cells. These findings raise the possibility that *opn* may be a *ras*-responsive gene which may contribute to the increase in metastatic ability of these tumor cells.

5.6 CONCLUSIONS AND SIGNIFICANCE

Oncogenes have been used extensively to study tumor formation and progression. Transformation of cells with a *ras* oncogene frequently, but not always, causes the cells to become metastatic. One such cell line that is sensitive to malignant conversion by *ras* is NIH 3T3. The metastatic ability of these cells correlates with p21 protein expression, and increases as expression of p21 increases. Expression of some genes is dramatically increased in *ras*-transformed cells, including *opn*. This observation has led to the proposal that *opn* may be involved in the increase in metastatic ability of *ras*-transformed NIH 3T3 cells, and that *opn* expression may correlate with both *ras* expression and the metastatic ability of these tumor cells.

The work presented here, and published in *Anticancer Research* (Chambers, Behrend, *et al.*, 1992b) and in *Clinical and Experimental Metastasis* in abstract format (Chambers, Behrend, *et al.*, 1990b), has demonstrated a positive correlation between *ras*, *opn*, and the metastatic ability of tumor cells. In addition, this work raises a number of questions regarding the regulation of *opn* gene expression by *ras*, and the role of OPN in tumor progression and metastasis. Guo *et al.* (1995) have addressed the issue of *opn* gene regulation by *ras*, and chapter 6 of this thesis addresses the role of OPN in malignancy.

CHAPTER 6

CONTRIBUTION OF OSTEOPONTIN TO THE TUMORIGENIC AND METASTATIC PROPERTIES OF *RAS*-TRANSFORMED NIH 3T3 CELLS

6.1 BACKGROUND

OPN is an $\alpha_v\beta_3$ integrin-binding secreted phosphoprotein that has been implicated in a number of physiological, developmental, and pathological processes, including cancer. In the mid to late 1980's, several different research teams have identified OPN independently on the basis of its frequent association with the transformed phenotype. Senger *et al.* (1979; 1980; 1983; 1985) reported that a number of phosphoproteins (58-62 kD) were over-expressed in transformed mammalian cell lines relative to the non-transformed counterparts. Chackalaparampil *et al.* (1985) reported a similar secreted phosphoprotein, pp69, in Rous sarcoma virus-transformed rat cells. Independently, Smith and Denhardt (1987) identified an mRNA species, designated "2ar", in JB6 epidermal cells based on its inducibility by the tumor promoter TPA. "2ar", the major secreted phosphoprotein of many transformed rodent cell lines, was soon demonstrated to be the mouse homologue of rat osteopontin (Craig *et al.*, 1988). Expression of *opn* was shown to be induced by TPA in mouse epidermis *in vivo*, and to correlate with tumor progression *in vivo* (Craig *et al.*, 1989; 1990). Further evidence for an association of OPN with malignancy was provided by the observation that OPN could be induced by transformation with oncogenes, such as *ras* or *myc* (Craig *et al.*, 1988; Castagnola *et al.*, 1991). *Opn* mRNA was increased in metastatic, *ras*-transformed cells relative to non-transformed control cells (Craig *et al.*, 1990).

Work discussed in chapter 5 of this thesis and published in *Anticancer Research* (Chambers, Behrend, Wilson, and Denhardt, 1992b) has demonstrated a strong correlation between *opn* expression, *ras* expression, and the metastatic potential of 6 individual *ras*-transformed cell lines. Both OPN protein and mRNA levels were increased in *ras*-transformed NIH 3T3 cells, particularly when the cells were selected for enhanced metastatic ability. This strong correlation between *opn*, *ras*, and the metastatic potential of *ras*-transformed cells has raised the question whether OPN contributes functionally to the development of a malignancy.

6.2 OBJECTIVE

A number of independent reports reviewed above (section 6.1 and section 1.5.12), identifying OPN in conditions of transformation and malignancy, had demonstrated an association between over-expression of OPN and malignancy. However, no report had demonstrated that expression of OPN by tumor cells contributed functionally to the development of the transformed state and progression to a more malignant phenotype. The objective of this study was, therefore, to determine whether OPN produced by tumor cells contributed functionally to the tumorigenic and metastatic properties of tumor cells.

6.3 EXPERIMENTAL DESIGN

This study involved the manipulation of OPN expression levels in metastatic, *ras*-transformed NIH 3T3 cells (PAP2) by expression of antisense *opn* RNA. Cells were transfected with a mammalian expression vector (pNMH-asOPN) that drives expression of antisense *opn* RNA from the heavy metal-inducible mouse metallothionein-I (MT-I) promoter (Khokha and Denhardt, 1987). Transfectants were analyzed for expression of antisense *opn* RNA, *opn* mRNA, and secretion of OPN protein *in vitro*. Transfectants were also analyzed for their tumorigenic and metastatic properties *in vivo*, to determine if these had changed as a result of the manipulations with antisense *opn* RNA.

6.4 THE EXPRESSION VECTORS pNMH AND pNMH-asOPN

The expression vector used in this study was pNMH which was constructed by Dr. Rama Khokha and used successfully in the down-regulation of TIMP (tissue inhibitor of metalloproteinases) in Swiss 3T3 cells (Khokha and Denhardt, 1987; Khokha *et al.*, 1989). pNMH was constructed from pMT-I/hGH (Stout *et al.*, 1985) and pIFPneo, a derivative of pSV2neo (Southern and Berg, 1982) and contains two transcriptional units. One unit encodes resistance to the neomycin analogue Geneticin G-418 sulfate under control of the SV40 early promoter, and uses termination and polyadenylation signals from SV40. The second unit contains the mouse metallothionein-I (MT-I) promoter which drives expression of any cDNA inserted downstream into a *Bam*HI site, and termination and polyadenylation signals from the human growth hormone gene.

The section of the mouse MT-I promoter that is present in pNMH is composed of a ~770 bp fragment of sequence 5′ to the mouse MT-I gene (Stout *et al.*, 1985). MT gene expression is inducible by heavy metals such as cadmium (Cd), zinc (Zn), copper (Cu), and iron (Fe), with Cd and Zn being the most potent inducers (Andrews, 1990). Metal inducibility is mediated via *cis*-acting binding sequences termed metal response elements (MREs), five of which have been identified in the murine MT-I promoter region (-63 to -187) (Stuart *et al.*, 1984). All five murine MREs are functional, but differ in their activity and act co-operatively in metal-induced expression of the MT gene (Stuart *et al.*, 1984; Palmiter, 1987). A number of proteins have been described that bind to metal-responsive DNA sequences, suggesting that metals do not directly bind to MREs but rather have a regulatory role (Heuchel *et al.*, 1994). In support of this notion Palmiter (1994) hypothesized the existence of a zinc-sensitive inhibitor that interacts with the MRE-binding transcription factor MTF-1 (Westin and Schaffner, 1988), where zinc functioned to release MTF-1 from the inhibitor. In addition to being inducible by heavy metals, MT gene expression can also be induced by other agents including: (1) steroid hormones such as glucocorticoids or progesterone, (2) cytokines such as interleukins and interferons, (3) tumor promoters such as TPA, (4) bacterial endotoxin, and (5) conditions of stress including inflammation (reviewed in Andrews, 1990). Furthermore, numerous Sp1 binding sites have been located in the MT-I promoter of different species, and recently an antioxidant response element was identified in the mouse MT-I promoter (Palmiter, 1987; Dalton *et al.*, 1994).

The expression vector pNMH-asOPN contained the full-length *opn* cDNA cloned into the *Bam*HI site downstream of the MT-I promoter in an antisense orientation (Craig, 1989). This construct was then transfected into PAP2 cells with the goal of examining the effects of altered *opn* expression on the behavior of the cells.

6.5 RESULTS

6.5.1 Isolation and Screening of Transfected Clones

Transfection of PAP2 cells with pNMH or pNMH-asOPN was carried out as described in chapter 3 section 3.3. Transfection of PAP2 cells with the control vector

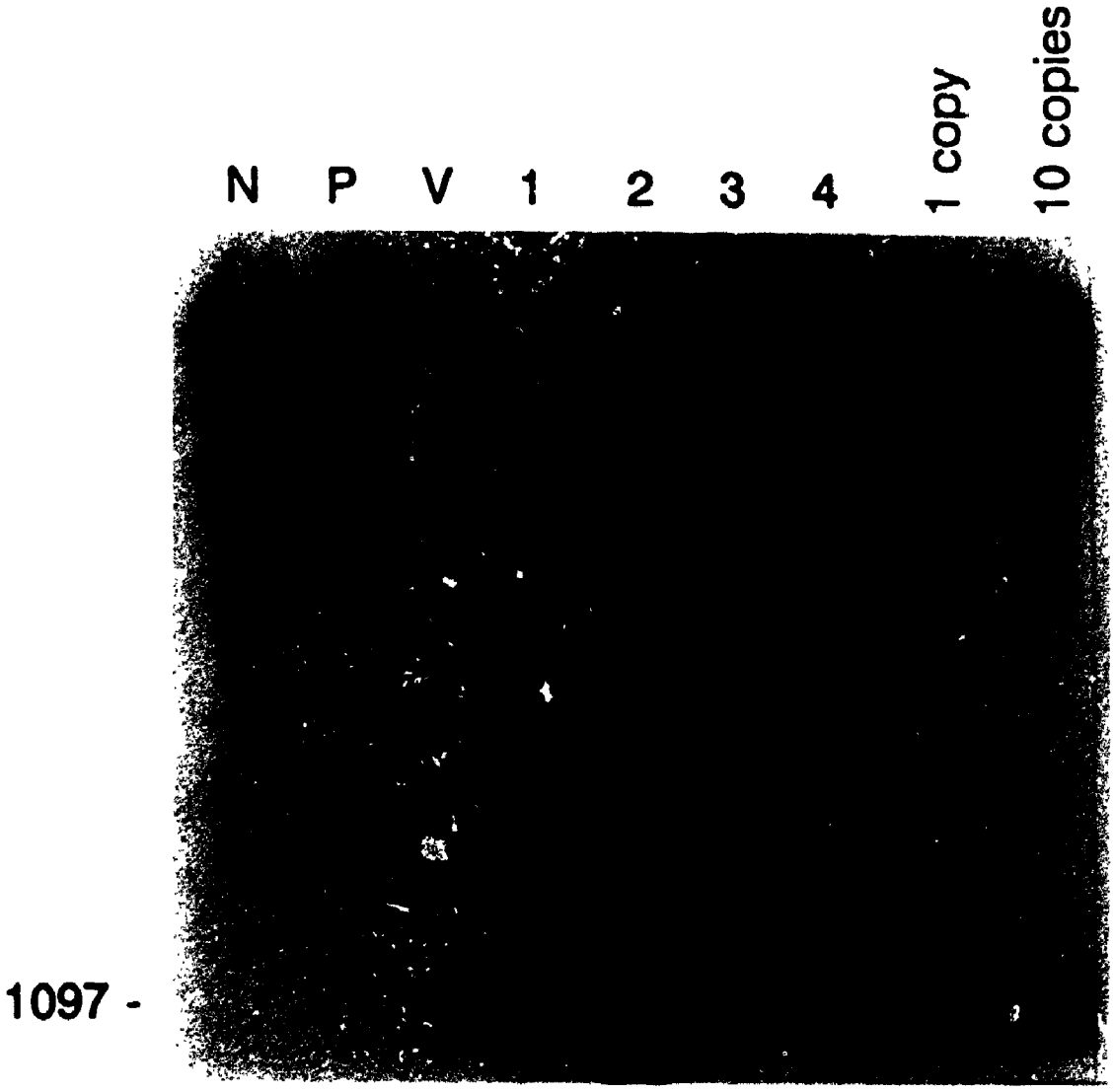
pNMH yielded 22 G418-resistant colonies of transformants, while transfection with pNMH-asOPN yielded 23 G418-resistant colonies on one plate and 9 on a second plate. No colonies were observed on the control plate of PAP2 cells not treated with DNA. Five colonies were picked randomly from each of the 2 experimental plates transfected with pNMH-asOPN, and 5 from the control plate transfected with pNMH. In addition, a pooled population of all colonies on each plate was also obtained. Cells were briefly grown in medium plus 200 μg (active/ml) Geneticin, and a stock of cells was frozen.

From the stock of 10 frozen asOPN transfectants, 4 clones (2 from each plate transfected with antisense *opn* DNA) were selected randomly and analyzed by Southern blot analysis for the presence of the transfected *opn* DNA (Figure 6.1). NIH 3T3, PAP2, and the vector control cell lines served as negative controls. On this Southern blot, the *upper* band in each lane represents the endogenous *opn* gene. The band labeled *1097 bp* represents the transfected antisense *opn* DNA. Clones asOPN1 and asOPN4 had incorporated ~ 1 copy/cell of the transfected *opn* DNA, while asOPN2 had incorporated > 10 copies/cell, and asOPN3 had incorporated < 1 copy/cell of the transfected antisense *opn* DNA. asOPN1 and asOPN2 are two distinct clones as denoted by their unique banding patterns. The band above the *1097 bp opn* DNA in lanes 1 and 2, as well as the band above the endogenous *opn* gene in lane 2, likely represent multiple integration sites of the transfected *opn* DNA. asOPN3 and asOPN4 may represent the same clone based on their identical banding pattern (a single, identical integration site) and on the fact that these clones were derived from the same transfection plate. The observation that asOPN3 incorporated < 1 copy/cell and asOPN4 incorporated 1 copy/cell suggests that either asOPN3 is not clonal in origin and distinct from asOPN4, or that asOPN3 started out as clonal and changed during development.

6.5.2 Expression of Antisense *opn* RNA in Cells Transfected with pNMH-asOPN and Grown *in vitro*

Purification of RNA from cells grown *in vitro*, RNA gel electrophoresis, Northern blot analysis, and hybridization of Northern blots were performed as described in chapter 3 sections 3.4.1, 3.5, 3.6, and 3.8, respectively. Analysis of the expression of antisense

Figure 6.1. Southern blot analysis of DNA from PAP2-derived clones transfected with pNMH-asOPN. DNA (10 μg/lane) digested with *Bam*HI was separated, blotted and probed with an oligolabeled *opn* (*2ar*) cDNA probe as described in the "Materials and Methods". pNMH-asOPN DNA equivalent to 1 and 10 copies of the plasmid per genome, plus 10 μg PAP2 DNA, were digested with *Bam*HI and electrophoresed together with the samples to determine copy number per cell of the transfected plasmid (pNMH-asOPN). Lanes correspond as follows: *N*, NIH 3T3; *P*, PAP2; *V*, Vector control; *1*, asOPN1; *2*, asOPN2; *3*, asOPN3; *4*, asOPN4. The *upper band* in each lane represents the endogenous *opn* gene. The band labeled *1097 bp* in lanes labeled *1* through *4* represents the transfected antisense *opn* DNA. The same-sized bands (*1097 bp*) in lanes labeled *1 copy* and *10 copies* represent 1 and 10 copies of the transfected antisense *opn* DNA per PAP2 genome, respectively (Behrend *et al.*, 1994).


N
P
V
1
2
3
4
1 copy
10 copies
1097 -

opn RNA, *opn* mRNA, *ras* mRNA, and *18S* rRNA was carried out on the same set of 7 cell lines that were used for Southern blot analysis. Cells were assessed for expression of these genes in uninduced (Figures 6.2B) and cadmium chloride ($CdCl_2$)-induced conditions (Figure 6.2A). RNA from NIH 3T3 cells served as negative control, RNA from PAP2 cells as positive control, and RNA from the vector transformant served as vector control. asOPN1 and asOPN2 expressed undetectable and low levels of antisense *opn* RNA, respectively, in the absence of $CdCl_2$ (Figure 6.2B), and low and high levels of antisense *opn* RNA, respectively, in the presence of $CdCl_2$ (Figure 6.2A). Neither asOPN3 nor asOPN4 expressed detectable levels of antisense *opn* RNA under either condition. The levels of *opn* mRNA expression of all 4 antisense *opn*-transfectants were high in the presence and absence of $CdCl_2$ (except asOPN4, which had slightly reduced levels of *opn* mRNA in the absence of $CdCl_2$; Figure 6.2B), and were comparable to the levels of *opn* mRNA expression of the parent PAP2 cells and the vector control cells. Expression of *ras* mRNA was low in NIH 3T3 cells, high in PAP2 and vector control cells, as expected (see chapter 5, Figure 5.1), and high in all 4 antisense *opn* transfectants in the presence and absence of $CdCl_2$. Analysis of the levels of *ras* mRNA is an important control since reduced expression of *ras* could result in reduced expression of *opn*. Expression of *18S* rRNA was comparable in all lanes, confirming equal loading of RNA samples.

6.5.3 Levels of OPN Protein Secretion in Antisense *opn* RNA-Expressing Cells Grown *in vitro* and induced with cadmium chloride

Metabolic radiolabeling of secreted proteins, barium citrate precipitation of ^{35}S-labeled proteins, and SDS-PAGE analysis were performed as described in chapter 3 sections 3.9, 3.10, and 3.11, respectively. SDS-PAGE analysis of ^{32}P-labeled (Figure 6.3A) and ^{35}S-labeled (Figure 6.3B) secreted proteins from cells grown *in vitro* and induced with cadmium chloride was carried out to determine the levels of OPN protein secreted by the antisense *opn*-transfected cells when grown *in vitro*. ^{32}P-labeling of secreted proteins allowed for detection of phosphorylated OPN protein, while ^{35}S-labeling allowed analysis of the levels of non-phosphorylated OPN protein. Triplicate samples

Figure 6.2. Northern blot analysis of RNA from PAP2-derived transformants (A) with and (B) without cadmium chloride induction. Total cytoplasmic RNA (10 μg/ lane) was separated, blotted, and probed as described in the "Materials and Methods". Lanes are as follows: *N*, NIH 3T3; *P*, PAP2; *V*, Vector control; *1*, asOPN1; *2*, asOPN2; *3*, asOPN3; *4*, asOPN4. The same blot was sequentially stripped and reprobed with the oligonucleotide probes *ras*, *opn*, *as opn*, and *18S* as described in the "Materials and Methods". The oligonucleotide probe *opn* specifically recognizes and hybridizes to "sense" *opn* mRNA, whereas *as opn* specifically recognizes and hybridizes to "antisense" *opn* RNA. RNA sizes are: *ras*, 1.3-kilobases (kb); *opn*, 1.6-kb; *as opn*, 1.6-kb; and *18S*, 2.1-kb (Behrend *et al.*, 1994).

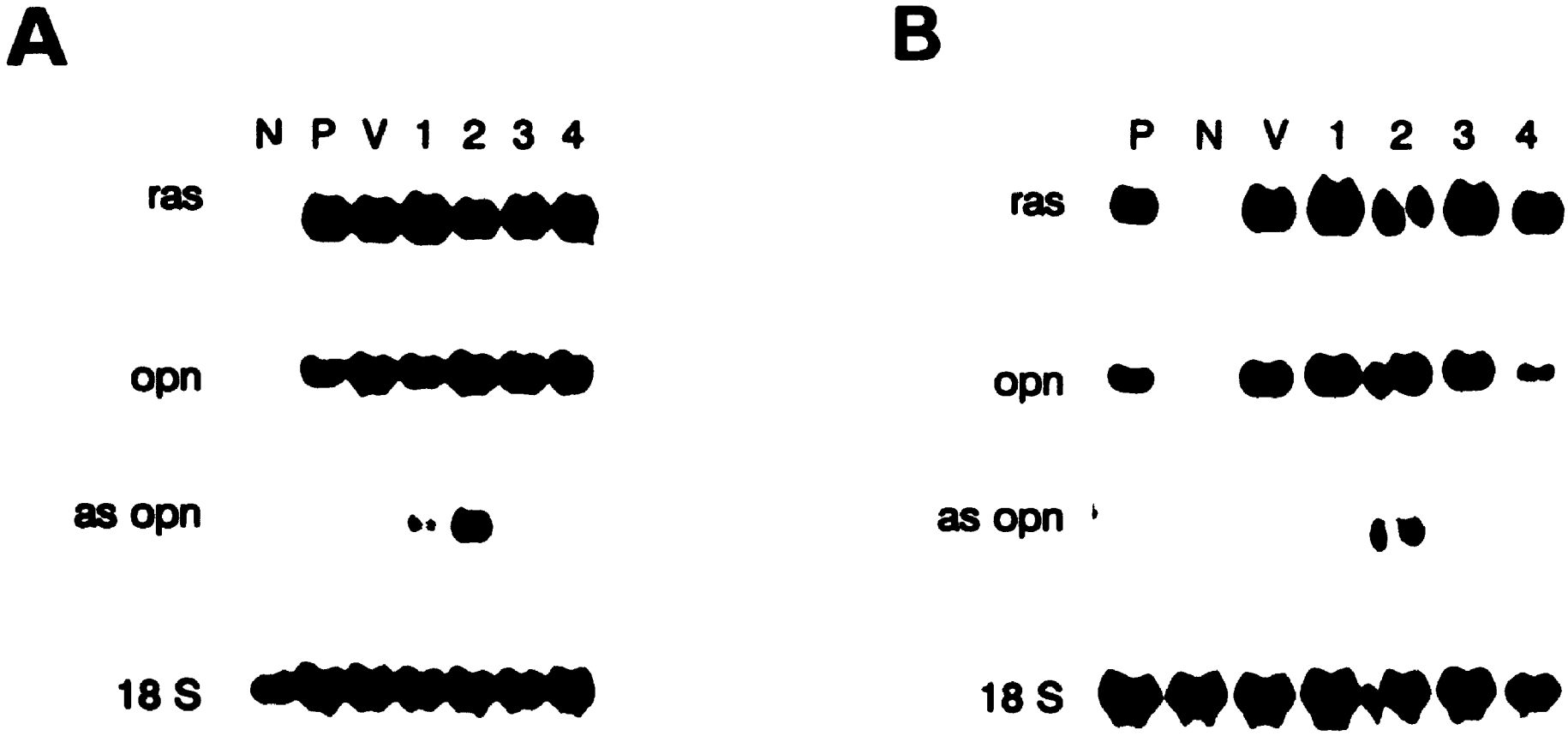

A
N P V 1 2 3 4
ras
opn
as opn
18 S
B
P N V 1 2 3 4
ras
opn
as opn
18 S

Figure 6.3 (A). SDS-polyacrylamide gel electrophoresis of ^{32}P-labeled secreted proteins. Cells were induced with 20 μM $CdCl_2$ and metabolically labeled with[^{32}P]-orthophosphate, and medium was electrophoresed on a 12% SDS-polyacrylamide gel as described in the "Materials and Methods". Triplicate samples of ^{32}P-labeled medium were electrophoresed for each cell line. The band labeled *69 kD* represents the OPN protein. Volumes loaded per lane were standardized to an equal number of labeled cells. Lanes correspond as follows: *N*, NIH 3T3; *P*, PAP2; *V*, Vector control; *1*, asOPN1; *2*, asOPN2; *3*, asOPN3; *4*, asOPN4 (Behrend *et al.*, 1994).

A

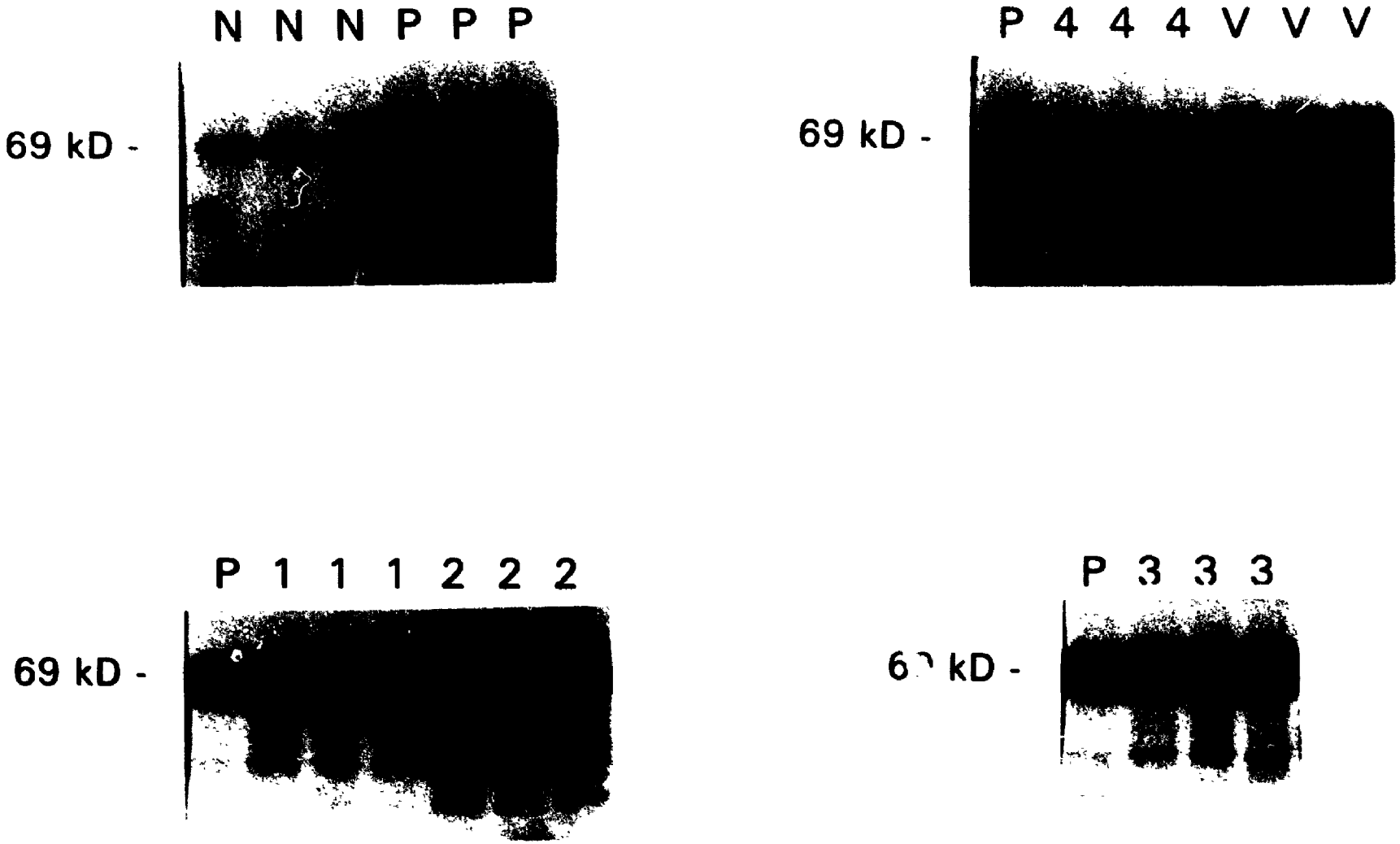
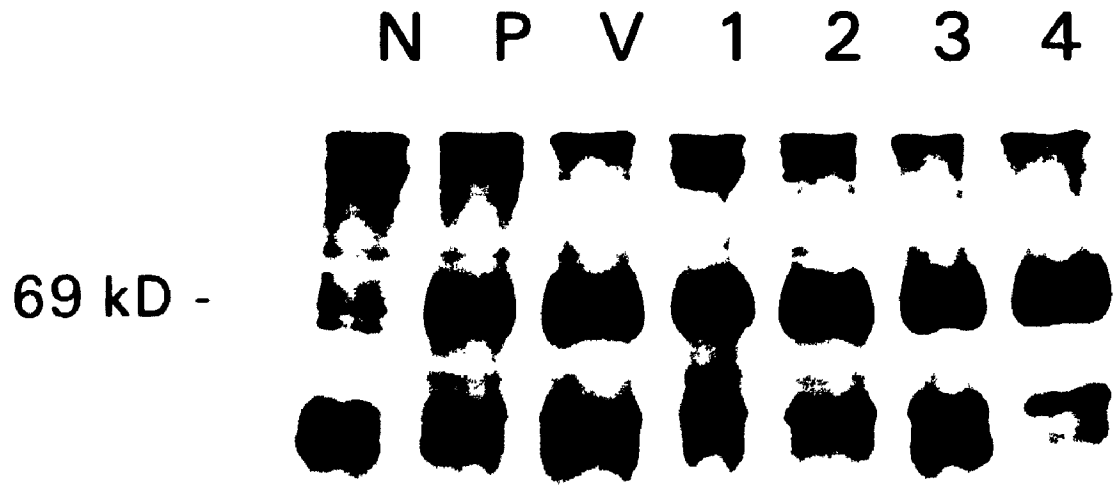


Figure 6.3 (B). SDS-polyacrylamide gel electrophoresis of ^{35}S-labeled secreted proteins. Cells were induced with 20 μM $CdCl_2$ and metabolically labeled with Tran^{35}S-Label, and medium was electrophoresed on a 12% SDS-polyacrylamide gel as described in the "Materials and Methods". A single sample of ^{35}S-labeled medium was analyzed for each cell line. ^{35}S-labeled medium was precipitated with barium citrate prior to electrophoresis. The band labeled *69 kD* represents the OPN protein. Volumes loaded per lane were standardized to an equal number of labeled cells. Lanes correspond as follows: *N*, NIH 3T3; *P*, PAP2; *V*, Vector control; *1*, asOPN1; *2*, asOPN2; *3*, asOPN3; *4*, asOPN4 (Behrend *et al.*, 1994).

B



of ^{32}P-labeled secreted phosphoproteins were analyzed, while only a single sample of ^{35}S-labeled secreted proteins was analyzed. NIH 3T3 cells secreted low levels of ^{32}P-labeled OPN protein of an apparent molecular weight of 69-kD, while PAP2 cells secreted high levels of OPN protein (Figure 6.3A). The vector control and all 4 antisense *opn*-transfected clones secreted equal or slightly higher amounts of ^{32}P-labeled OPN protein compared to PAP2 cells (Figure 6.3A). Similar results were observed for ^{35}S-labeled secreted proteins (Figure 6.3B). Small amounts of ^{35}S-labeled OPN protein were secreted by NIH 3T3 cells, while equally increased amounts of ^{35}S-labeled OPN protein were detected for PAP2, vector control, and all 4 antisense *opn*-transfected clones when grown *in vitro*. The band labeled *69-kD* identifies OPN based on previous characterization by immunoprecipitation and barium citrate precipitation (Craig *et al.*, 1988).

In these experiments the apparent molecular weight of OPN was 69-kD, compared to 51-kD and 58-kD in Figures 5.3 and 5.4, respectively. Two variables were different in the SDS-PAGE gels shown in Figures 6.3A and 6.3B compared to those of Figure 5.3: (1) the cell number plated per well during the metabolic radiolabeling, and (2) a cross-linking agent. For the results illustrated in Figures 5.3, 5.0 x 10^5 cells/well were plated, while for the results illustrated in Figures 6.3A and 6.3B, 3.5 x 10^5 cells/well were plated. it has not been previously reported that the apparent molecular weight of the OPN protein varied with the cell density, but it is possible that changes in post-translational modification during more stressful growth conditions such as higher cell density may account for this phenomenon, although this hypothesis needs further testing. In addition, the cross-linking agent piperazine di-acrylamide (PDA) (BioRad) was used in place of methylene-bis-acrylamide (bis) (BioRad) for the SDS-PAGE gels shown in Figure 6.3A and 6.3B. Although the manufacturer (BioRad) claims that PDA can be substituted for bis on a gram-for-gram basis, the possibility that a different cross-linking agent may have affected the migration of the OPN protein in an SDS-PAGE gel can not be excluded.

6.5.4 Metastatic Ability of Antisense *opn* RNA-Expressing Cells *in vivo* in the Chick Embryo

Analysis of all 7 cell lines for their metastatic ability *in vivo* in the chick embryo is illustrated in Figure 6.4, and was carried out as described in chapter 3 section 3.12. Metastatic ability was assayed by means of the ouabain plating assay, which makes use of the natural resistance of rodent cells to ouabain cytotoxicity relative to chick embryo cells. As expected, NIH 3T3 cells were non-metastatic as measured by the inability to recover cells from chick livers. PAP2 cells were metastatic, showing a range of metastatic ability from very high to very low, reflecting the heterogeneity of this polyclonal cell population. The vector control cell line was highly metastatic as measured by $> 10^3$ cells recovered per chick liver. asOPN3 was as highly metastatic as the vector control cell line, and asOPN4 was as metastatic and as heterogeneous in its range of metastatic ability as PAP2. It is noteworthy that neither asOPN3 nor asOPN4 expressed antisense *opn* RNA (cf. Figure 6.2). asOPN1 and asOPN2 expressed antisense *opn* RNA (cf. Figure 6.2) and were poorly metastatic in this assay. The differences between asOPN1 or asOPN2 and either PAP2 or vector were statistically significantly different as assayed by the Mann-Whitney Rank Sum test (see Table 6.1 for the P-values of the statistical analysis).

6.5.5 Tumorigenicity and Metastatic Ability of Antisense *opn* RNA-Expressing Cells *in vivo* in the Nude Mouse

Clones asOPN1 and asOPN2, as well as PAP2 and vector were analyzed further for their tumorigenic (Figure 6.5) and metastatic properties (Figure 6.6) in nude mice as described in detail in chapter 3 section 3.13 and 3.14.

Figure 6.5 illustrates the tumorigenicity of PAP2 cells, the vector control cell line, asOPN1, and asOPN2 in nude mice as assayed by subcutaneous (s.c.) injection of $1x10^5$ cells into the right hind thigh of groups of 5 mice each. Tumor growth was measured as length x width over time. PAP2 cells and the vector control cell line started forming primary tumors in each of the 5 mice 7 days post-injection. These tumors increased rapidly in size until they reached a cross-sectional area of ~ 5 cm^2, at which

Figure 6.4. Experimental metastatic ability of PAP2-derived clones in the chick embryo. Cell lines (under non-induced conditions) were assayed for their metastatic potential by i.v. injection of $1x10^5$ cells into the chorioallantoic membrane veins of 11-day-old chick embryos as described in the "Materials and Methods". Each *circle* represents the number of viable rodent cells recovered from a single embryonic liver days after injection. Lanes are as follows: *N*, NIH 3T3; *P*, PAP2; *V*, Vector control; *1*, asOPN1; *2*, asOPN2; *3*, asOPN3; *4*, asOPN4. asOPN1 and asOPN2 were statistically significantly different from PAP2, Vector control, asOPN3, and asOPN4 by the Mann-Whitney Rank Sum Test (Behrend *et al.*, 1994).

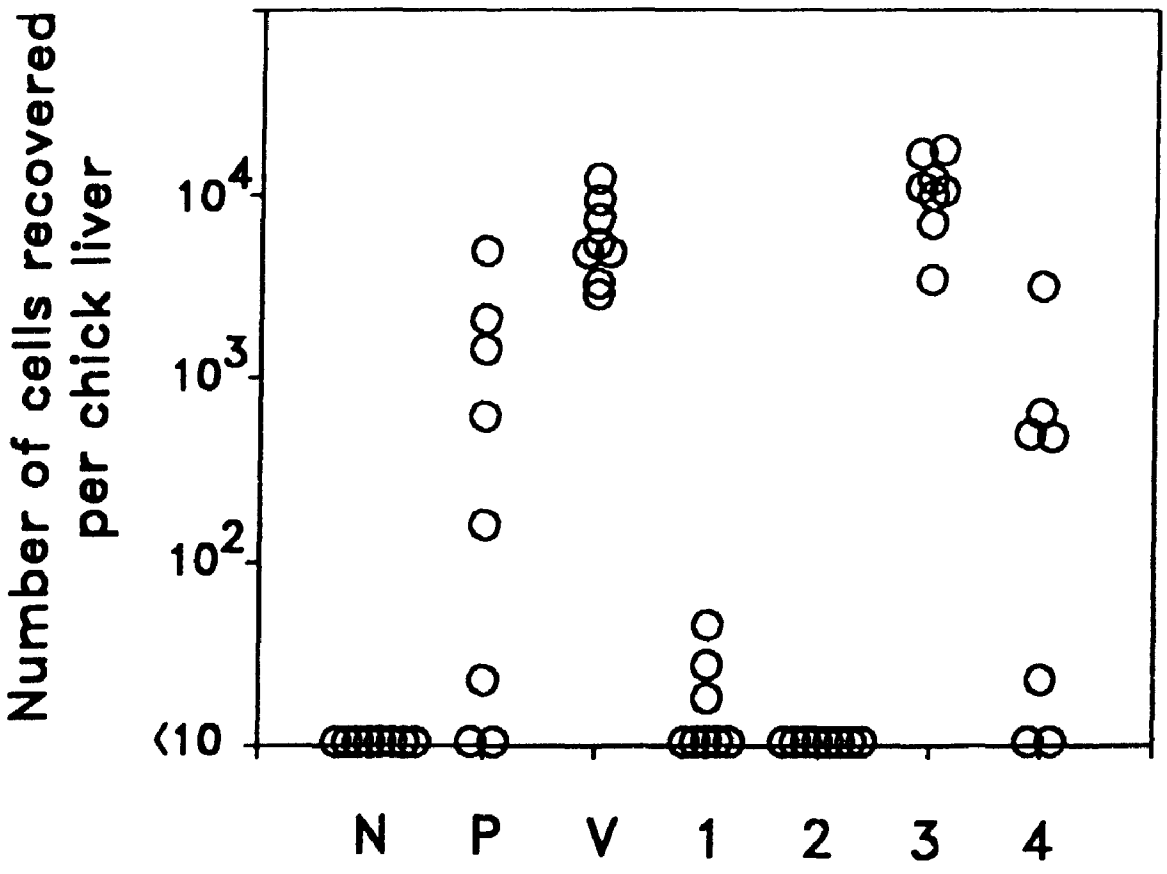

Number of cells recovered per chick liver
10^4
10^3
10^2
<10
N
P
V
1
2
3
4

Table 6.1

Statistical Analysis of Data from the Chick Embryo Metastasis Assay

by the Mann-Whitney Rank Sum Test

Cell Lines Compared[a]	Statistical Difference ?	P-Value[c]
PAP2 vs. asOPN1	Yes	0.0499
PAP2 vs. asOPN2	Yes	0.0104
PAP2 vs. asOPN3	Yes	0.0003
PAP2 vs. asOPN4	No	0.7790
Vector vs. asOPN1	Yes	0.0002
Vector vs. asOPN2	Yes	0.0002
Vector vs. asOPN3[b]	Yes	0.0333
Vector vs. asOPN4	Yes	0.0006
asOPN1 vs. asOPN2	No	0.2340
asOPN1 vs. asOPN3	Yes	0.0002
asOPN1 vs. asOPN4	Yes	0.0401
asOPN2 vs. asOPN3	Yes	0.0002
asOPN2 vs. asOPN4	Yes	0.0004
asOPN3 vs. asOPN4	Yes	0.0003
PAP2 vs. Vector[b]	Yes	0.0014

[a] Values for NIH 3T3 cells were the same as for asOPN2

[b] The normality test was passed, therefore the t-test applied

[c] A P-value of $P \leq 0.05$ indicates a statistically significant difference; a P-value of $P \geq 0.05$ indicates no statistically significant difference

Figure 6.5. Tumorigenicity of PAP2-derived clones in nude mice. Tumor growth rates were assayed by s.c. injection of $1x10^5$ cells (under non-induced conditions) into the right hind thigh of nude mice as described in the "Materials and Methods". Five mice were injected per cell line. Each symbol represents the growth of a tumor in a single mouse. For asOPN1, the symbols overlap since no tumor grew in any of the five mice (Behrend *et al.*, 1994).

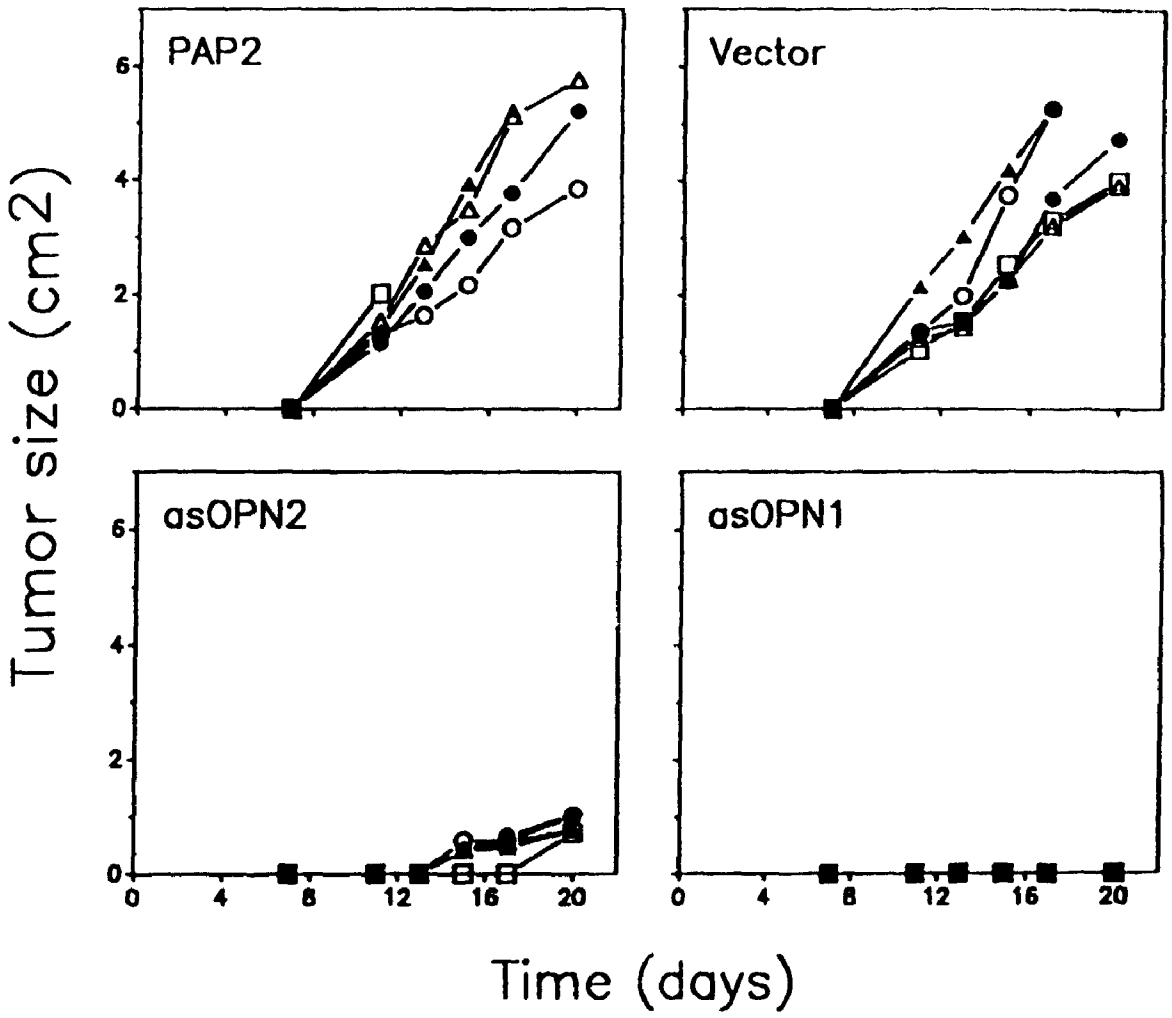
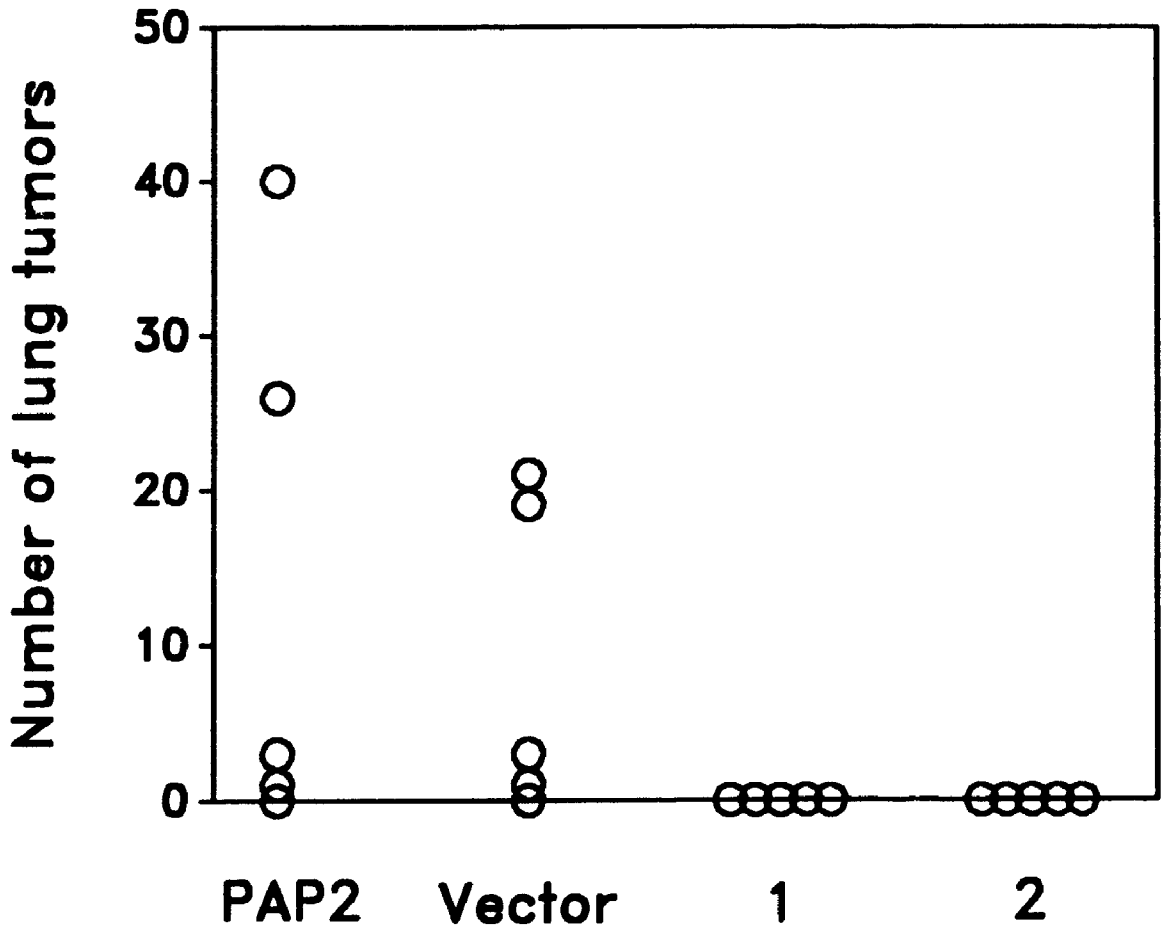

PAP2
Vector
asOPN2
asOPN1
Tumor size (cm2)
Time (days)
0
2
4
6
0
4
8
12
16
20

Figure 6.6. Experimental metastatic ability of PAP2-derived clones in nude mice. Cell lines (under non-induced conditions) were assayed for their metastatic potential by i.v. injection of $1x10^5$ cells into the tail vein of nude mice as described in the "Materials and Methods". Five mice were injected per cell line. Each *circle* represents the number of lung tumors detected in an individual mouse. Cell lines are as follows: *PAP2*; *Vector*; *1*, asOPN1; *2*, asOPN2. asOPN1 and asOPN2 were statistically significantly different from PAP2 and Vector by the Mann-Whitney Rank Sum Test (Behrend *et al.*, 1994).


Number of lung tumors
50
40
30
20
10
0
PAP2
Vector
1
2

time the mice were sacrificed and the tumors were dissected. In contrast, asOPN1 produced no tumors in any of the 5 mice during the assay period (22 days), while asOPN2 produced small, slower growing tumors only after an extended lag period of about a week (13 days post-injection) (Figure 6.5).

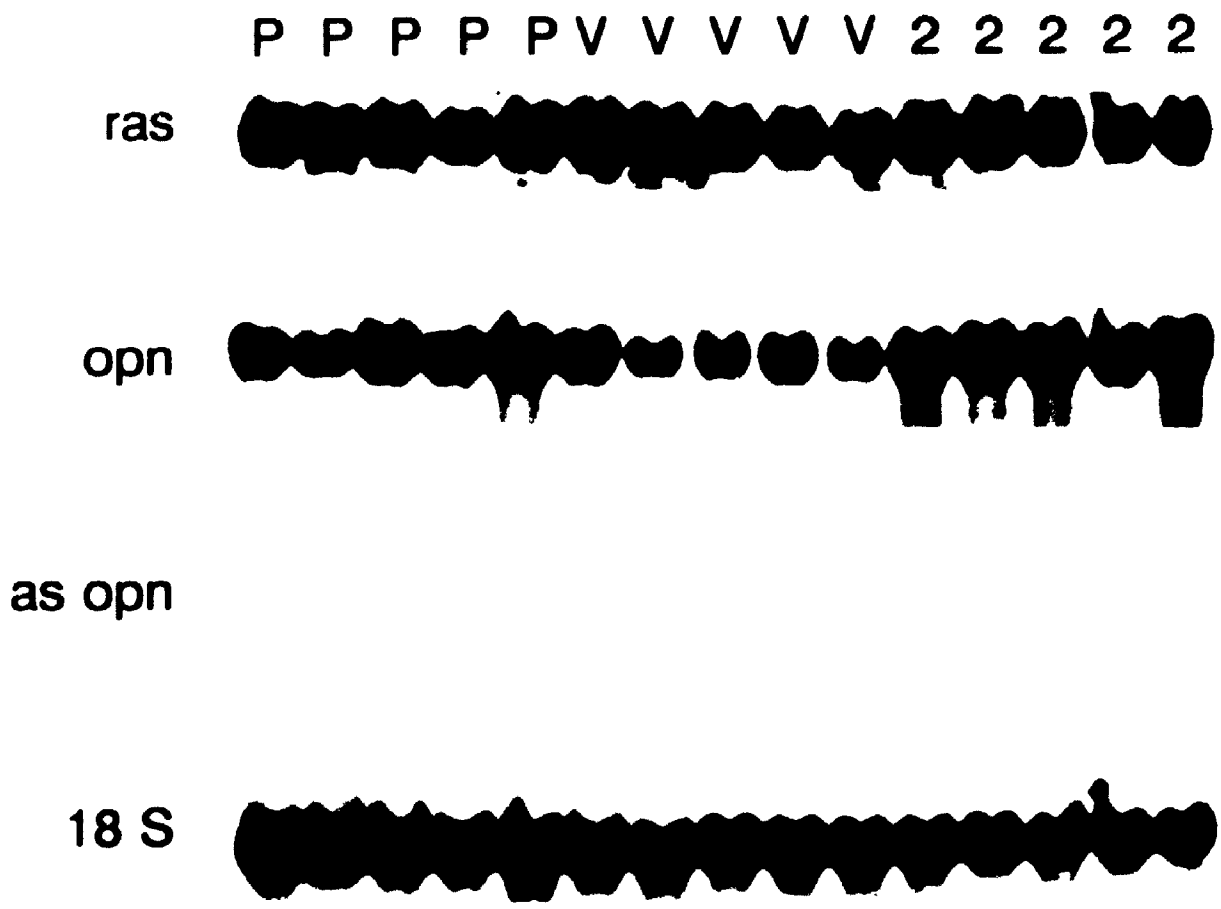
Analysis of the metastatic ability of PAP2 cells, the vector control cell line, asOPN1, and asOPN2 in nude mice is shown in Figure 6.6 and was measured by counting the numbers of lung metastases formed four weeks after intravenous (i.v.) injection of $1x10^5$ cells into the tail vein of nude mice. The results in Figure 6.6 showed that PAP2 cells and the vector control cell line were metastatic and heterogeneous in their range of metastatic ability. asOPN1 and asOPN2, on the other hand, did not form any lung metastases and were non-metastatic in nude mice (Figure 6.6). Analysis of these data by the Mann-Whitney Rank Sum test demonstrated that the differences between asOPN1 or asOPN2 and either PAP2 or the vector control cell line were statistically significant ($P=0.0317$ for all comparisons).

NIH 3T3, asOPN3, and asOPN4 cell lines were not tested for their tumorigenic and metastatic abilities in nude mice, since NIH 3T3 have previously been shown to be non-tumorigenic in mice (Chambers *et al.*, 1990a; Tuck *et al.*, 1991), and the metastatic potential for asOPN3 and asOPN4 have already been established in the chick embryo assay.

6.5.6 Expression of Antisense *opn* RNA in Primary Mouse Tumors *in vivo*

Primary tumors were generated by s.c.injection of groups of 5 mice with PAP2 cells, the vector control cell line, and clone asOPN2 (see chapter 3 section 3.14). RNA from primary tumors was analyzed for *in vivo* expression of *opn* mRNA and antisense *opn* RNA to determine if RNA expression levels of these cells *in vivo* (Figure 6.7) differed from the expression levels of these cells when grown *in vitro* (cf. Figure 6.2). Expression of *opn* mRNA *in vivo* was high in tumors of PAP2 and asOPN2 cells and slightly lower in tumors of vector control cells, but still comparable to *opn* mRNA levels expressed from these cells *in vitro* (cf. Figure 6.2). Expression of antisense *opn* RNA *in vivo* was not detected in any of the five tumors derived from asOPN2 cells even after

Figure 6.7. Northern blot analysis of RNA extracted from primary tumors. Total RNA (10 μg/lane) was extracted, separated, blotted, and probed as described in the "Materials and Methods". RNA from 5 individual tumors was analyzed for each cell line. Each lane represents the RNA isolated from one individual tumor. Lanes correspond as follows: *P*, PAP2; *V*, Vector control; *2*, asOPN2. The same blot was sequentially stripped and reprobed with the oligonucleotide probes *ras*, *opn*, *as opn*, and *18S* as described in the "Materials and Methods". The oligonucleotide probe *opn* specifically recognizes and hybridizes to "sense" *opn* mRNA, whereas *as opn* specifically recognizes and hybridizes to "antisense" *opn* RNA. RNA sizes are: *ras*, 1.3-kilobases (kb); *opn*, 1.6-kb; *as opn*, 1.6-kb; and *18S*, 2.1-kb (Behrend *et al.*, 1994).


P P P P PV V V V V 2 2 2 2 2
ras
opn
as opn
18 S

long exposure of the film (Figure 6.7), in contrast to the high levels of antisense *opn* RNA expression by these cells when grown *in vitro* (cf. Figure 6.2). Expression of *ras* mRNA was high in all tumors, and hybridization with *18S* rRNA confirmed equal loading of all lanes.

6.5.7 Analysis for the Presence of Transfected Antisense *opn* DNA Copies in Primary Mouse Tumors *in vivo*

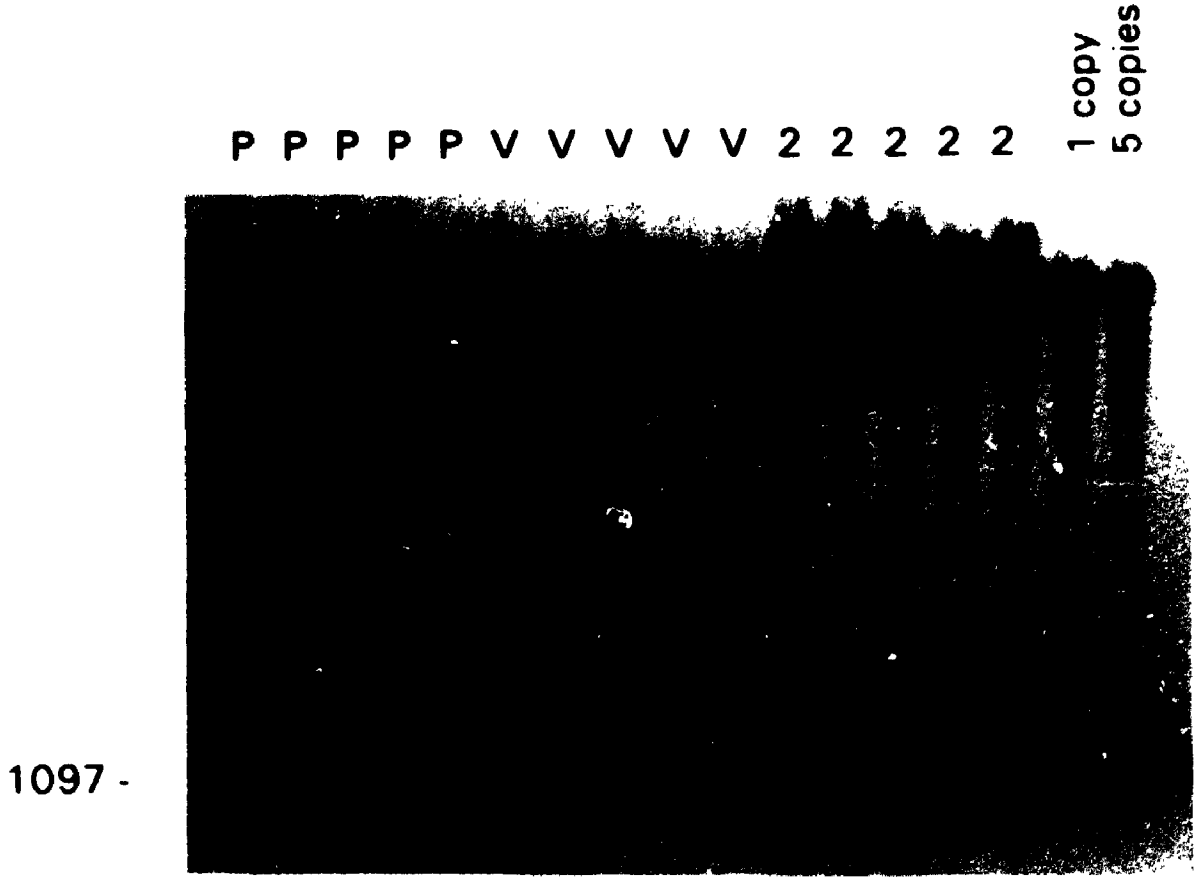
Primary tumors were generated as described above (section 6.5.6; and chapter 3 section 3.14). DNA from 5 individual tumors of each of PAP2, vector control, and asOPN2 cells was analyzed by Southern blot analysis to determine the copy number per genome of the transfected antisense *opn* DNA in asOPN2 after growth *in vivo* (Figure 6.8), as compared to the copy number in these cells when grown *in vitro* (cf. Figure 6.1). The *upper* band in each lane represents the endogenous *opn* gene. The band labeled *1097 bp* represents the transfected antisense *opn* DNA. Four individual tumors of asOPN2 had ~1 copy/cell of the transfected antisense *opn* DNA present, whereas 1 tumor contained <1 copy/cell, in contrast to the >10 copies/cell present in these cells growing *in vitro* just prior to *in vivo* injection (cf. Figure 6.1).

6.6 DISCUSSION

Tumorigenesis and metastasis are complex, multi-step processes, in which the controlled expression of many genes is deregulated. OPN is a protein that has been associated with malignancy on a number of occasions. To clarify whether OPN contributes functionally to the development of a malignancy, antisense *opn* RNA was expressed in *ras*-transformed NIH 3T3 cells (PAP2) and the tumorigenic and metastatic properties of these cells were analyzed.

Two independent clones of antisense *opn*-transfected PAP2 cells have been identified, asOPN1 and asOPN2, that express antisense *opn* RNA *in vitro* when induced with cadmium chloride (Figure 6.2A), and slightly lower levels when not induced (Figure 6.2B). When these cells were tested *in vivo* for their tumorigenic and metastatic properties, both asOPN1 and asOPN2 were poorly tumorigenic and poorly metastatic

Figure 6.8. Southern blot analysis of DNA extracted from primary tumors. DNA (10 μg/lane) extracted from primary tumors was digested with *Bam*HI, separated, blotted, and probed with an oligolabeled *opn* (*2ar*) cDNA probe as described in the "Materials and Methods". DNA from 5 individual tumors was analyzed for each cell line. Each lane represents the DNA isolated from one individual tumor. pNMH-asOPN DNA equivalent to ' and 5 co; ies of the plasmid per genome, plus 10 μg PAP2 DNA, was digested with *Bam*HI and electrophoresed together with the other DNA samples to determine copy number per cell of the transfected plasmid (pNMH-asOPN). Lanes are as follows: *P*, PAP2; *V*, Vector control; *2*, asOPN2. The *upper band* in each lane represents the endogenous *opn* gene. The band labeled *1097 bp* in all five lanes labeled *2* (asOPN2) represents the transfected antisense *opn* DNA. The same-sized bands (*1097 bp*) in lanes labeled *1 copy* and *5 copies* represent 1 and 5 copies of the transfected antisense *opn* DNA per PAP2 genome, respectively (Behrend *et al.*, 1994).


P P P P P V V V V V 2 2 2 2 2
1 copy
5 copies
1097 -

(Figures 6.4 to 6.6), in contrast to two clones which did not express antisense *opn* RNA and which were tumorigenic and metastatic (Figure 6.4). These results suggest that cells expressing antisense *opn* RNA *in vivo* at the time of injection, and possibly thereafter, were significantly less malignant than the metastatic PAP2 and the vector control cell lines. The analyses for tumorigenicity and metastatic ability in this study were performed in nude mice which are immuno-compromised by virtue of being athymic. Analysis of malignant behavior *in vivo* using syngeneic mice may well have yielded a similar response, since adult nude mice used in this study contain a significant level of natural killer (NK) cell activity in spite of the lack of functional T-lymphocytes. NK cell activity is a basic immunological defense mechanism effective against bacteria and tumor cells. However, syngeneic mice do not constitute an appropriate assay system, since by virtue of being immuno-competent they may prevent tumor formation of even the control cells (PAP2 and vector control). The nature of the hypothesis under question in this study called for an assay system in which tumor formation could easily be achieved, since the objective was to observe lack of tumor formation in antisense *opn*-expressing cells.

In contrast to the poorly malignant behavior of asOPN1 and asOPN2 *in vivo*, analysis of *opn* mRNA and OPN protein levels *in vitro* showed that they were as high in asOPN1 and asOPN2 as they were for the two clones that did not express antisense *opn* RNA, and as high as for PAP2 and the vector control (Figures 6.2 and 6.3), even though the *in vivo* behavior of asOPN1 and asOPN2 in an animal host differed.

It appears that the levels of antisense *opn* RNA expressed by asOPN1 and asOPN2 *in vitro* were not sufficient to down-regulate the overall levels of *opn* mRNA and OPN protein *in vitro*. It is possible that the antisense *opn* RNA expressed by asOPN1 and asOPN2 *in vitro* did down-regulate *opn* mRNA expression and OPN protein secretion in a subset of asOPN1 and asOPN2 cells, but not in other subsets of these clones, resulting in no reduction in the overall levels of *opn* mRNA and OPN protein. Alternatively, expression of *opn* may be so critical to the maintenance of the transformed phenotype and the survival of the tumor cells, that any attempt to down-regulate *opn* is counteracted by increased expression of the gene. It is also possible that duplex formation between antisense *opn* RNA and *opn* mRNA may have stabilized the *opn* mRNA

by some as yet unknown mechanism, although RNA:RNA duplexes are generally believed to induce cleavage of the mRNA by RNase III (Zhang and Roth, 1994).

The significantly reduced tumorigenic and metastatic potentials of asOPN1 and asOPN2 *in vivo*, in the presence of an apparently contradictory lack of reduced *opn* expression *in vitro*, could be accounted for by a number of possible explanations.

Shortly after injection of asOPN1 and asOPN2 into the animals, cells expressing antisense *opn* RNA may have died, since presumably their non-malignant phenotype did not favor cell growth and survival of the cell line. Alternatively, cells expressing antisense *opn* RNA may have been impaired in their ability to grow, and thereby may have given cells not expressing antisense *opn* RNA or cells that had eliminated expression of antisense *opn* RNA a selective growth advantage. This hypothesis assumes that the injected cell populations of asOPN1 and asOPN2 cells were heterogeneous in composition and/or evolved into heterogeneous cell populations during growth *in vivo*, an assumption that is consistent with Nowell's clonal evolution theory (1976) and other hypotheses of dynamic heterogeneity of tumor cells (Harris *et al.*, 1982; Heppner and Miller, 1983; Ling *et al.*, 1985). Consequently, asOPN2 cells expressing antisense *opn* RNA, and impaired in their ability to grow or destined to die, may have been overgrown by asOPN2 cells not expressing antisense *opn* RNA or asOPN2 cells that had lost expression of antisense *opn* RNA. Selection for cells not expressing antisense *opn* RNA may then have given rise, after a lag period (Figure 6.5), to tumors that contained cells which lacked expression of the antisense *opn* RNA (Figure 6.7) and which contained fewer copies of the transfected antisense *opn* DNA (Figure 6.8).

asOPN1 cells, in contrast to asOPN2 cells, never formed visible primary tumors in the time the mice were assayed (~ 3 weeks) (Figure 6.5). Clones asOPN1 and asOPN2 are two independent, distinct clones, and are not expected to behave exactly alike. It is possible that the time period of 3 weeks was insufficient for asOPN1 cells not expressing antisense *opn* RNA to emerge, however, the same explanations apply to the observations made for this clone.

The mechanisms by which expression of antisense *opn* RNA may have resulted in reduced tumor formation and reduced metastatic ability of asOPN1 and asOPN2 cells,

possibly by affecting the cells' ability to grow or to die, may involve OPN-specific or OPN-independent mechanisms.

(1) It is possible that more efficient down-regulation of *opn* mRNA expression and OPN protein secretion was accomplished in asOPN1 and asOPN2 cells grown *in vivo* as compared to *in vitro*, based on the nature of the MT-I promoter. Factors such as metals, interleukins, interferons, and glucocorticoid hormones are present in mice and are known to drive expression of the MT-I promoter used in this study (Andrews, 1990). The *in vivo* levels of antisense *opn* RNA expression by asOPN1 and asOPN2 cells may have been sufficiently high to down-regulate expression of *opn* mRNA and OPN protein in the animals. Reduced production of OPN protein early after injection of the cells into the animals would coincide with the period of low malignancy. As expression of antisense *opn* RNA would be lost with successive proliferation in conditions lacking the selective pressure to retain the expression vector, cells not expressing antisense *opn* RNA would emerge and proliferate to form a tumor. A number of reports support the hypothesis that metallothionein promoters may function more efficiently *in vivo* than *in vitro*. It has been shown that iron and copper are effective inducers of metallothionein *in vivo*, but poor or ineffective inducers in chicken primary hepatocyte cultures (Andrews, 1990). In addition, the chicken MT gene is hormonally responsive *in vivo*, but not in primary cultures of embryonic fibroblasts or hepatocytes (Fernando and Andrews, 1989; Fernando *et al.*, 1989). Finally, it has been reported that macrophage-derived cytokines such as interleukin-1α, interleukin-1β, interleukin-6, tumor necrosis factor-α, and interferons can dramatically induce MT gene expression *in vivo*, but interleukin-1α induced MT gene expression only poorly in cultured human cells (Karin *et al.*, 1985; Andrews, 1990). Regulation of MT gene expression is a complex process, which often involves cooperative interactions of inducers, a condition that is difficult to mimic in culture. For example, interleukin-6 appears to exert a direct effect on hepatic MT gene expression *in vivo*, but induces MT gene expression *in vitro* only in the presence of glucocorticoids and zinc (Schroeder and Cousins, 1990). It appears therefore that MT gene regulation involves multiple independent, but synergistic pathways.

Murphy *et al.* (1994) have shown recently that both human MT-IIA mRNA levels

and MT protein levels were significantly increased in A431 squamous carcinoma cells exposed to hypoxia, conditions of low oxygen levels, and subsequent reoxygenation. Analysis of a 0.2-kb fragment of the human MT-IIA proximal promoter for transcriptional activity revealed an increase in transcriptional activity in response to hypoxic stress and reoxygenation, suggesting the existence of a hypoxic response element in the proximal promoter of the human MT-IIA gene (Murphy *et al.*, 1994). In addition, Schwarz *et al.* (1995) have evidence that the mouse MT-I promoter is up-regulated by hypoxia, possibly through the AP-1 site in its promoter region. Tumor cells injected into mice are likely to encounter conditions of hypoxia while trying to establish a tumor. Hypoxic conditions, in addition to hormones, metals, and inflammatory mediators, may induce the MT-I promoter to high levels and reduce OPN expression in asOPN1 and asOPN2 cells *in vivo*, resulting in conditions of low malignancy (Figures 6.4, 6.5, 6.6) that can only be overcome after expression of antisense *opn* RNA has been lost.

(2) The possibility that a protein encoded in the antisense *opn* DNA strand was responsible for the observed reduction in tumorigenic and metastatic properties was excluded by virtue of an analysis of the antisense *opn* DNA strand for potential open reading frames (ORFs) and a comparison of these potential ORFs with known proteins. Two potential open reading frames, 109 and 87 amino acids in length, were identified in the antisense *opn* DNA strand by nucleic acid coding sequence analysis using the method of Fickett (PC/Gene 6.5; Swiss-Prot 17; EMBL 25; IntelliGenetics, Inc., Mountain View, CA; Fickett, 1982). A comparison of the two potential ORFs with known proteins using the updated GenPep database of the BLAST network service of the National Center for Biotechnology Information revealed that the stretches of similarity between these two potential ORFs and known proteins were too short and were not significant. The absence of any message in mouse tumors upon hybridization with the probe *as opn* to detect antisense *opn* RNA (Figure 6.7) suggests that under normal physiological conditions, no RNA is transcribed from the non-coding (antisense) strand of the endogenous *opn* gene, refuting the possibility that the antisense *opn* DNA encodes a protein that may be responsible for the reduced malignancy reported for these cells.

(3) The possibility that the observed reductions in tumor formation and metastatic

ability were due to clonal heterogeneity of individual PAP2 cells cannot be excluded, but is considered unlikely, based on experience with clones of PAP2 cells. Individual clones of PAP2 cells with reduced tumorigenic and metastatic properties have only been observed when expression of *ras* mRNA was significantly reduced in the cells (chapter 5, Figure 5.2; Hill *et al.*, 1988), and clearly in the cells discussed here (asOPN2 cells, Figure 6.7) expression of *ras* mRNA was high.

(4) It is also possible that expression of antisense *opn* RNA in asOPN1 and asOPN2 cells may have affected the expression of other cellular genes. Although antisense RNA is designed to specifically inhibit the expression of a chosen target gene, there is no guarantee, in any experiment using antisense RNA, that the effect of the antisense RNA is limited to the specific target mRNA. Too little is known about the mechanisms of action of antisense RNA, and the possibility that antisense RNA may also affect the expression of genes other than the one it was targeted to cannot be excluded.

(5) Furthermore, it cannot be excluded that the lack of continuous selective pressure to retain the expression vector, such as the continuous culture of transfectants in Geneticin/G-418 when cells were grown *in vitro*, may account for the loss of expression of antisense *opn* RNA and reduced copy number of the transfected antisense *opn* DNA observed after growth of the cells *in vivo*. It is not known whether loss of antisense *opn* RNA expression is an active process mediated by the tumor cells, or a passive process that results from growth of the cells *in vivo* in the absence of any selective pressure to retain the expression vector. However, regardless of the mechanism of loss of antisense *opn* RNA expression, the fact remains that expression of antisense *opn* RNA early after injection is associated with reduced malignancy, and tumor formation is associated with loss of antisense *opn* RNA expression.

The results presented in this study suggest that OPN secreted by tumor cells is advantageous to the malignant phenotype, and indicate a functional role for OPN in malignancy. Recently, two independent reports have confirmed the findings presented in this study (Gardner *et al.*, 1994; Su *et al.*, 1995). Gardner *et al.* (1994) have generated two clones of high OPN-producing, malignant B77-Rat1 fibroblast cells that expressed antisense *opn* RNA. The two clones showed a 50% and 75% decrease in OPN

production *in vitro*, and were reduced in their ability to form lung tumors in nude mice after intravenous injection, and colonies in soft agar. Furthermore, these antisense transfectants showed increased spreading on vitronectin. Su *et al.* (1995) transfected TPA-promotable JB6 epidermal cells with an expression vector that contained the *opn* cDNA in antisense orientation and under control of a dexamethasone-inducible MMTV-LTR promoter. Four stable antisense transfectants showed reduced OPN production *in vitro* in the presence of dexamethasone and TPA and failed to form colonies in soft agar. The reasons why these two studies showed reduced OPN protein production in antisense *opn*-transfected cells grown *in vitro* in contrast to the findings described here may be several-fold. First, all three studies used different promoter constructs for the production of antisense *opn* RNA. Gardner *et al.* (1994) utilized the constitutive SV40 early promoter, and Su *et al.* (1995) employed the glucocorticoid-inducible MMTV-LTR promoter, which are both very strong promoters. In these studies, the heavy metal-inducible mouse MT-I promoter was utilized which has been used successfully to down-regulate expression of TIMP in Swiss 3T3 cells (Khokha and Denhardt, 1987; Khokha *et al.*, 1989). However, as discussed earlier in this chapter, the metallothionein promoters are very complex promoters which have not yet been fully characterized, and which appear to exhibit complex, synergistic regulation mechanisms (Andrews, 1990). Another reason for the successful down-regulation of OPN protein production by Gardner *et al.* (1994) and Su *et al.* (1995) may lie in the amounts of antisense *opn* RNA produced by the individual clones. Particularly for the antisense *opn* transfectants generated by Gardner *et al.* (1994) expression of antisense *opn* RNA was readily detectable, and was equal to or higher than expression of the control gene G3PDH. House-keeping genes such as G3PDH or 18S are usually expressed at high levels in cells. However, effective down-regulation of genes does not always depend on an excess of antisense RNA produced relative to the levels of mRNA. Effective down-regulation of c-*fos* was accomplished with low levels of detectable antisense c-*fos* RNA in a system that utilized the glucocorticoid-inducible MMTV promoter (Nishikura and Murray, 1987). The same promoter was used by Su *et al.* (1995), who reported down-regulation of OPN protein in the presence of low to moderate levels of detectable antisense *opn* RNA relative to the

actin control gene. Another factor that likely accounts for the lack of down-regulation of OPN protein production by cells grown *in vitro* reported in this study is the fact that only 4 randomly-selected antisense transfectants were analyzed, compared to a greater number of transfectants analyzed by Gardner *et al.* (1994) and Su *et al.* (1995).

However, the reports by Gardner *et al.* (1994) and Su *et al.* (1995) have confirmed the findings first demonstrated in this study, that OPN contributes functionally to the malignant properties of tumor cells.

6.7 CONCLUSIONS AND SIGNIFICANCE

The work discussed in chapter 5 of this thesis, demonstrating a strong correlation between expression of *opn*, expression of *ras*, and the metastatic properties of *ras*-transformed NIH 3T3 cells, gave rise to the hypothesis that OPN may contribute functionally to the tumorigenic and metastatic properties of cancer cells.

The strategy chosen in this study to demonstrate a functional role for OPN in malignancy was to manipulate *opn* mRNA and OPN protein levels by expressing antisense *opn* RNA in metastatic *ras*-transformed NIH 3T3 cells and examining the effects of this manipulation on the malignant properties of the cells.

Two independent clones (asOPN1 and asOPN2) were identified that expressed antisense *opn* RNA *in vitro* (Figure 6.2), and that had significantly reduced tumorigenic and metastatic properties *in vivo*, compared to two other clones that were metastatic and that did not express antisense *opn* RNA (Figures 6.4, 6.5, 6.6). Surprisingly, the levels of *opn* mRNA and OPN protein produced by asOPN1 and asOPN2 when grown *in vitro* did not differ from those of controls and those of two metastatic clones that did not express antisense *opn* RNA (Figures 6.2, 6.3), even though the *in vivo* behavior of asOPN1 and asOPN2 differed (Figures 6.4, 6.5, 6.6).

These results demonstrate that expression of antisense *opn* RNA by asOPN1 and asOPN2 at the time of injection and possibly thereafter are associated with reduced tumorigenicity and reduced metastatic ability of these cells. Only after expression of antisense *opn* RNA was independently eliminated from asOPN2 cells in 5 individual mice, did tumors begin to form (Figure 6.5, 6.7). Although no reduction in OPN pro-

duction was observed when the cells were grown *in vitro*, this does not exclude the possibility that OPN protein levels may have been reduced *in vivo*, possibly due to the MT-I promoter functioning more efficiently *in vivo* than *in vitro*. These results provide indirect evidence to support the hypothesis that OPN contributes functionally to the malignant properties of tumor cells. The decrease in malignancy of the cells (asOPN1 and asOPN2) was not due to a protein encoded in the antisense *opn* DNA, and likely not due to clonal variation of individual PAP2 cells. However, effects of the antisense *opn* RNA on other genes or cellular processes can not be ruled out.

Other conclusions that can be drawn from this study include the realization that gene expression pattern observed for cells grown *in vitro* cannot confidently be extrapolated to the gene expression pattern observed *in vivo*. Furthermore, we can conclude that much remains to be learned about the mechanisms of action of antisense RNA. The work discussed here is published in the journal *Cancer Research* (Behrend *et al.*, 1994).

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

OPN began its "début" as a secreted phosphoprotein that was over-expressed in many transformed mammalian cell lines (Senger *et al.*, 1979). In 1985, it was independently identified as one of several phosphoproteins of mineralized tissues (Franzén and Heinegård, 1985). The discovery that the major secreted phosphoprotein of transformed cell lines was identical to bone-derived OPN (Craig *et al.*, 1988) represented a significant milestone in the study of this protein. Now, eight years later, the physiological function of this protein is still unknown, but much progress has been made in our knowledge of this protein. OPN has been identified in many cells, tissues, and body fluids, and has been implicated in many normal and pathological processes, including: cell adhesion, cell signaling, the regulation of bone metabolism, inhibition of kidney stone formation, nephritis, vascular disease, calcium metabolism, the immune system, and cancer.

The study presented in this thesis has contributed to our knowledge of OPN in three ways. First, this thesis has established the correct gene structure for mouse *opn*. The gene structure of mouse *opn* was in dispute as a result of two conflicting mouse *opn* gene sequences that had been reported in the literature. By resolving this conflict, and by establishing the correct mouse *opn* gene structure, this portion of the thesis has set the stage for future studies on the regulation of *opn* gene expression, and made a significant contribution to a better understanding of OPN. Secondly, on several independent occasions, over-expression of OPN had been reported to be associated with transformation and malignancy. Analysis of the levels of *opn* mRNA and OPN protein produced by a series of *ras*-transformed NIH 3T3 cells has demonstrated that levels of *opn* mRNA and OPN protein correlated with the levels of *ras* mRNA expression and with the metastatic potential of the cells. This finding represented a significant milestone in the study of OPN, since it formed the basis for two important hypotheses about OPN: (1) that *opn* may be a *ras*-induced gene that is subject to direct regulation by RAS; and (2) that OPN may functionally contribute to the tumorigenic and metastatic properties of *ras*-transformed cells. Hypothesis 1 was confirmed by Guo *et al.* (1995) with the identi-

fication of a RAS-activated enhancer (RAE) element in the mouse *opn* promoter. Hypothesis 2 was addressed in the third section of this thesis, and evidence was presented that supports a functional role for OPN in malignancy. The objective of the third section of this thesis was to decrease OPN levels in metastatic, *ras*-transformed NIH 3T3 cells, using antisense *opn* RNA, and to examine the effects of this alteration on the tumorigenic and metastatic properties of the cells. The results showed that cells expressing antisense *opn* RNA *in vitro* were significantly impaired in their ability to form primary tumors and metastases *in vivo*. Furthermore, primary tumors formed only after an extended lag period, and after expression of the antisense *opn* RNA was completely abolished. Although the levels of *opn* mRNA and OPN protein were not visibly reduced in the cells when grown *in vitro*, the possibility remained that the metallothionein-I (MT-I) promoter used in this study to drive expression of the antisense *opn* RNA may have been used more efficiently *in vivo* than *in vitro*. However, other mechanisms that may explain the reduced malignancy of the antisense *opn* RNA-expressing cells can not be excluded. These findings were confirmed recently by two reports demonstrating that cells down-regulated for OPN using antisense *opn* RNA have reduced tumorigenicity (Gardner *et al.*, 1994; Su *et al.*, 1995).

7.2 Future Directions

Studies examining the regulation of *opn* gene expression are already underway. Denhardt and Guo (1993) had previously reported a number of potential transcription factor binding sites in the mouse *opn* promoter, the correct location of which has been confirmed by the analysis presented in this thesis. Analysis of *opn* gene regulation by Guo *et al.* (1995) has identified a novel *ras* activated enhancer, distinct from known *ras* response elements, in the promoter of the mouse *opn* gene. This enhancer has been reported to interact with a putative ETS-related transcription factor whose activity correlates with the metastatic potential of the cell (Guo *et al.*, 1995). Further analysis of the *opn* gene and promoter region will likely identify many additional regulatory elements, and will improve our understanding of the regulation of this gene.

To further clarify the role OPN plays in malignancy, the two clones asOPN1 and

asOPN2 that are reduced in their tumor-forming and metastatic abilities *in vivo*, could be analyzed by intravital videomicroscopy (IVVM). IVVM offers the opportunity to visualize cell behavior *in vivo*, and may offer clues as to the fate of the cells after they have been injected into the animals.

In addition, these clones might be tested for their performance in an *in vivo* angiogenesis assay. OPN is a ligand for the integrin $\alpha_v\beta_3$, whose expression on vascular tissue has been reported to be increased during angiogenesis (Brooks *et al.*, 1994a). Ligation of $\alpha_v\beta_3$ has also been reported to be required for the survival and maturation of newly forming blood vessels (Brooks *et al.*, 1994b). It would be of interest to determine whether tumor-derived OPN protein might play a role in promoting angiogenesis.

In addition, it may be of interest to determine if clones asOPN1 and asOPN2 might undergo apoptosis more readily *in vivo* than control PAP2 cells. Apoptotic death may be one possible mechanism of eliminating antisense *opn* RNA-expressing cells *in vivo* so that only cells not expressing antisense *opn* RNA may give rise to a tumor.

Another approach to demonstrate that OPN contributes functionally to malignancy would involve expression of OPN in cells that are tumorigenic but non-metastatic, and that do not usually produce OPN. Increased production of OPN may supply these cells with the stimulus that is needed for progression to a metastatic phenotype. Irrespective of the fact that in many human cancers macrophages surrounding tumor foci are the predominant source of OPN, it would be worthwhile to determine the effects of over-expressing OPN in tumorigenic, non-metastatic cells that usually do not express OPN. The contribution of OPN to malignancy may be a cell type-specific phenomenon, since in cancers of the kidney and the endometrium tumor cells were positive for *opn* mRNA (Brown *et al.*, 1994). It is possible that in cancers of different cell type proteins which are functionally redundant with OPN may execute the functions that OPN executes in cells where it is over-expressed.

It is possible that OPN functions to protect tumor cells from nitric oxide (NO)-mediated host cell cytotoxicity by inhibiting production of NO as proposed by Denhardt and Chambers (1994). However, it is also possible that by virtue of its ability to bind to integrin receptors, OPN may function as a signaling molecule to promote tumor cell

growth in an autocrine fashion. Binding of OPN to cells has been shown to alter intracellular calcium levels, which represent an integral component of cellular signaling pathways (Miyauchi *et al.*, 1991; 1993; Zimolo *et al.*, 1994). In addition, tumor cell-derived OPN protein may play a role in the formation of new blood vessels, since OPN has been shown to mediate endothelial cell and smooth muscle cell migration (Clyman *et al.*, 1992; Yue *et al.*, 1994; Liaw *et al.*, 1995). In this respect, OPN would significantly promote the development of secondary metastases by attracting newly-forming blood vessels to the primary tumor.

OPN was first identified as a secreted phosphoprotein that was over-expressed in many transformed mammalian cell lines. After two decades of intensive research, OPN is now known to be also produced by many normal cells and tissues, and to be involved in a number of normal and pathological processes, including cancer. While each new discovery about OPN seems to reveal another small piece of the mystery, it seems to raise more questions than it gives answers. However, as research on OPN continues, the significance of this protein to normal cell behavior and to the pathologies OPN has been associated with becomes increasingly evident. Elucidating the function of OPN will represent a significant contribution to our understanding of the processes that OPN is involved in, including cell adhesion, cell signaling, regulation of bone metabolism, inhibition of kidney stone formation, nephritis, vascular disease, the immune system, and cancer.

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